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## Doctor's Dissertation

A Study of Phenolic and Carbohydrate Materials in the newly formed Tissues of Aspenwood

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# A STUDY OF THE PHENOLIC AND CARBOHYDRATE MATERIALS IN THE NEWLY FORMED TISSUES OF ASPENWOOD

A thesis submitted by

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TABLE OF CONTENTS

# INTRODUCTION

Most studies of the chemical composition of wood have been concerned with its uses as a raw material. However, it is always interesting, and often useful, to understand the processes by which a raw material originates or is created. It is well known botanically that the annual increments of wood in a tree result from the activity of the cambium which is a thin, cylindrical layer of cells located between the outermost wood (xylem) and the innermost bark (phloem).

Although there has been much speculation about the chemistry of the transformations from cambium to wood, there have been only a few attempts at experimental analysis. This is reflected in the following statements.

Of cambial chemistry in general it has been said (1): "Unfortunately, the chemistry and biochemistry of the cambium layer have been investigated only slightly. Such investigations are at present highly needed."

As regards carbohydrates it has been said (2): "More detailed work on the composition of the hemicelluloses of cambium is desirable and might throw further light on the origin and subsquent fate of these comparatively neglected cell-wall constituents."

Furthermore, concerning lignin, it should be said that if a low molecular weight substance (or substances), giving lignin in the aging process could be discovered in developing tissues of wood, it would be a significant contribution to the knowledge of the structure of lignin and the process of lignification.

A clearer understanding of the biochemical mechanisms by which wood is formed should be valuable to the pulp and paper industry. It is anticipated that a study of the details of lignin formation and deposition will engender ideas leading to more effective delignification processes. Similarly, a better knowledge of the chemical architecture of the cell-wall carbohydrates should lead to a more purposeful processing of wood and an improved comprehesion of fiber properties. It is also expected that studies of the biogenesis of woody tissues will be helpful to the forest geneticist in his efforts to develop superior trees for the pulp and paper industry.

Present concepts about wood formation are due largely to the work of plant anatomists and plant physiologists. As with all plant metabolism, the organic substance of wood is derived ultimately from photosynthesis. Sugars, the first products of photosynthesis, are formed in the leaves and are then transported by the sap stream in the phloem to the cambial region. Here they are ultimately transformed, by mechanisms still not clearly understood, into xylem and phloem elements.

The cambial cells increase the girth of the tree by dividing tangentially. Each cambial initial has a wall of its own which is separated from the walls of adjacent cells by a plastic intercellular substance. This substance probably forms the first partition between the cambial mother and the derivative. Differentiation of the daughter cells is manifested in physiologicial and morphological

changes. Gradually elements having more or less specialized characteristics are evolved. The xylem derivatives become tracheary elements, fibers, and parenchyma cells; and the phloem derivatives differentiate to sieve elements, bast fibers, parenchyma cells, and scleroids. This specialization is accompanied by cell-wall changes such as enlargement, secondary thickening, and lignification.

Each differentiated cell consists of a secondary wall and a primary wall, and is separated from adjacent cells by an intercellular substance referred to as the true middle lamella. The secondary wall has a comparatively low lignin content, whereas the primary wall and intercellular substance are highly lignified.

Our present knowledge of cambial chemistry is based on the pioneering work of a very few individuals (2-6). These investigations, with but one exception (black spruce), have been limited to European and Australian woods. Furthermore, tissues were collected at only one time during the growing season--usually in the spring--and the conventional methods of chemical analysis available at the time were used.

Although the botanical definition of the cambium describes it as a single layer of cells, it is impossible to distinguish this particular layer from the eight to ten rows of daughter cells adjacent to it, even with the aid of a high powered microscope. For this reason the material referred to as cambium by previous investigators

has been defined operationally as: (a) a gelatinous material removed from the surface of a freshly peeled log by using the rounded edge of a spoon (2), (b) a slightly viscous layer on the inner surface of the bark and on the outer surface of a freshly peeled log (4, 5), and (c) thin strips of undifferentiated material five or six cells thick removed by a concave scraper (6). In each case an attempt was made to remove only undifferentiated tissue. Only in the last mentioned study, however, was the authenticity of the cambial material verified by microscopic examination.

Table I shows the species and tissues studied by previous workers in cambial chemistry.

TABLE I

SPECIES AND TISSUES PREVIOUSLY STUDIED

| | Fraxinus elatior | Ulmus sativa | Pinus sylvestris | Picea mariana | Eucalyptus regnans |
|---|---|---|---|---|---|
| Outer bark | | | | X | X |
| Inner bark | | | | X | X |
| Cambium | X | X | X | X | X |
| New wood | X | X | | X | X |
| Sapwood | X | X | X | X | |
| Heartwood | | | | X | X |
| Investigator | 2 | 2 | 2 | 4, 5 | 6 |

The table shows that: (a) for five species a comparison has been made between cambial tissues and mature wood by conventional analyses; (b) for two species a comparison has been made between cambial tissues and inner bark by conventional analyses; (c) of the five species studied three were hardwoods and two were softwoods; and (d) of the five species studied three were European, one was American, and one was Australian.

These analyses may be summarized briefly by saying that cambial tissues are characterized by high nitrogen and uronic anhydride values and low hexosan, pentosan, and lignin contents (2, 4, 5, 6,). High nitrogen and uronic anhydride indicate that the cambial material is rich in protein and pectin, respectively; this is typical of embryonic tissues in general. The low hexosan, pentosan, and lignin values indicate that the cell-wall constituents are laid down, for the most part, as the tissues develop.

Cambial saps are rich in sucrose (2, 3, 4, 7) and contain glucose and fructose as well (3, 8). Guaiacol and vanillin, either or both of which could be lignin precursors, are also present in some cambial saps (9).

It is evident that the chemistry of the newly formed tissues of woody plants is a relatively unexplored field.

The present investigation was limited to a study of the phenolic and carbohydrate materials in the developing tissues of quaking aspen* (Populus tremuloides). Phenolic and carbohydrate materials were chosen

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*Subsequently referred to as aspen.

because of their obvious relation to the lignin and holocellulose in mature wood. The choice of aspen was based on its availability and on its increasing importance as a commercial pulpwood in the Lake States region.

## PRESENTATION OF THE PROBLEM

The immediate objectives of this study were: (a) to collect and analyze cambial and near-cambial tissues representing successive stages of maturity at several intervals during the growing season, (b) to study the phenolic and carbohydrate components of the tissue extracts, and (c) to obtain more definite information as to the chemical composition of the cell wall during growth as an aid in establishing the location of the individual components within the cell wall. In a more general sense, the study was made to answer some of the questions regarding the biogenesis of woody tissues and to stimulate interest in and thought to the metabolic processes whereby relatively simple compounds are converted into the more complex components of mature wood.

Previous studies of cambial and near-cambial tissues have given us the general analytical picture presented in the first section of this report. It seemed that the objectives of this study could best be met by using techniques of collection and analytical methods which would give more detailed information on the composition of these tissues. This approach appeared to offer the most promise because of the development and refinement of new techniques, viz., paper partition and ion-exchange chromatography. The results of such analyses take on added significance when considered in the light of the concepts of plant histology, biochemistry, and physiology.

The first objective was determined by the following considerations.

It was apparent that an attempt to collect cambial tissues only would be extremely difficult. Even if microdissection methods could be adapted, the accumulation of a sufficient quantity of material for analysis would have required an unwarranted amount of time. Furthermore, interpretation of the analyses would be unsatisfactory in the absence of comparable data for tissues intermediate in maturity. Moreover, it is known that spring- and summerwood differ in chemical composition. This consideration has been neglected in previous studies in cambial chemistry which were based on material collected at only one time during the growing season. For these reasons the decision was made to collect and analyze tissues representing successive stages of maturity at several intervals during the growing season.

The second objective evolved from the consideration that the first step in establishing the pathway by which a given metabolic end-product is formed is the isolation of intermediates. Such substances are to be found among the simpler compounds in the aqueous and alcoholic extracts of developing tissues.

The third objective came from the consideration that the tree is the major raw material for the pulp and paper industry. Hence, the ultimate objective of any study concerning it should be the best and most complete utilization possible. It is becoming increasingly evident that, "---the best chemical utilization of wood is dependent not only upon a knowledge of the amounts of certain substances (lignin, cellulose, pentosans, etc.) in wood, but also upon where

they are located in the cell wall..." (10). It was thought that such information might be obtained from materials collected and analyzed in the manner indicated.

## GLOSSARY

A glossary of some of the terms used in this study is presented below.

SX: soft xylem; young, translucent tissue removed from freshly peeled log sections in 1954 and characterized by a faint Wiesner test for lignin.

54: 1954 sapwood; xylem elements produced from the cambium during the 1954 growing season.

53: 1953 sapwood; xylem elements produced during the 1953 growing season.

The numerals appearing after the tissue designations refer to the collection date.

1 = June 17 and 18, 1954

2 = June 28, 29, and 30, 1954

3 = July 20, 21, and 22, 1954

4 = August 16, 1954

XSc: xylem scrapings; very young tissue collected in June, 1955, and characterized by a negative Wiesner test for lignin.

Billet: section of the tree bole from which the tissue samples were collected.

Marc: extracted tissue.

True middle lamella: intercellular substance; strongly lignified pectic layer that cements the cell walls of mature cells together.

Compound middle lamella: the compound layer between the secondary

walls of mature cells; this layer consists of intercellular substance (the true middle lamella) and the primary walls on each side of it.

EAW (9:2:2): a chromatographic solvent system consisting of 9 parts ethyl acetate, 2 parts acetic acid, and 2 parts water by volume.

BPW (10:3:3): a chromatographic solvent system consisting of 10 parts butanol, 3 parts pyridine, and 3 parts water by volume.

BPW (6:4:3): a chromatographic solvent system consisting of 6 parts butanol, 4 parts pyridine, and 3 parts water by volume.

AHP: aniline hydrogen phthalate chromatographic spray for simple sugars and oligosaccharides.

# COLLECTION AND PREPARATION OF MATERIAL

## COLLECTION OF MATERIAL

### COLLECTION OF SOFT XYLEM, 1954 SAPWOOD, AND 1953 SAPWOOD TISSUES

All experimental investigations were carried out on newly formed tissues obtained from eleven different aspen trees (29 to 33 years in age) cut in township 38N, range 10E, section 8 located within the Rhinelander Paper Company Industrial Forest just south of Eagle River, Wisconsin. Materials were collected at four intervals during the 1954 growing season--June 17 and 18, June 29 and 30, July 20 and 21, and August 16. Three trees were felled during each of the first three intervals and two trees were felled on August 16.* In order to minimize changes that might have occurred in the young tissues, the following precautions were taken: (a) the collection of soft xylem and 1954 sapwood was carried out in the forest as soon as possible after the trees had been felled, and (b) the tissues were immersed in absolute methanol to stop enzyme action as soon as they were removed from the log.

Since experimental results are influenced so much by methods of collection, the source of each material must be clearly specified. In this study, the tissues representing successive growth stages were as follows: The soft xylem, "SX", was the thin layer of xylem

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* It should be pointed out that the necessarily limited number of trees that were used for each sample may not have compensated statistically for the variability of the aspen population.

removed from the face of the barked log; the 1954 sapwood, "54", was the more fully differentiated xylem located between the soft xylem and the 1953 growth ring; and the 1953 sapwood, "53", was the xylem located between the 1954 sapwood and the 1952 growth ring. These terms are delineated in greater detail in the description of the methods of separation that follows.

The tissues were obtained from the boles of the respective trees. As soon as each tree was felled, the branches were removed and the tree was topped. The bole was then cut into billets 18 to 24 inches long. The bark on the bottom two feet of each bole was difficult to remove so this part of the tree was not used. Small blocks cut from each tree were preserved in 70% ethanol and sent to the Fiber Microscopy Department of The Institute of Paper Chemistry for sectioning and microscopic examination. A disk from the base of each tree was used for basal diameter and age determinations.

The bark was stripped from each billet in turn and the soft, succulent material on the log immediately under the bark was removed by drawing a sharp jackknife lengthwise along the face of the log in such a way that the blade of the knife was nearly parallel to the log surface. In most cases this material could be obtained as thin, translucent tissue resembling the skin of an onion. This tissue was called soft xylem. Microscopic examination showed it to be composed of the true cambium and some partially differentiated xylem elements.

Immediately under the soft xylem was tissue that had been

formed earlier in the 1954 growing season and was therefore more fully differentiated. It was called 1954 sapwood. On the first two field trips the 1954 sapwood was so thin that it had to be removed with a jackknife, but thereafter it could be collected more easily with a drawshave. The 1954 sapwood was shaved off to a depth determined by the appearance of a very thin layer of pale-yellow tissue located at the boundary between the 1954 sapwood and the 1953 growth ring. Microscopic examination of the yellow tissue revealed that it was composed of 1953 summerwood tissue.

The 1953 growth ring, located just beneath the 1954 tissue, was collected with a drawshave. It was designated 1953 sapwood and was shaved off to a depth determined by the appearance of the same pale-yellow tissue. Since it was anticipated that the extractive content of the 1953 sapwood would be much less than that of the younger tissues, a considerably greater quantity of this material had to be collected to have sufficient extract with which to work.

After the first two field trips, it was found that the amount of 1953 sapwood extract obtained in the available time fell far short of the anticipated need. Hence, on the following field trips the billets from which the soft xylem and 1954 sapwood had been removed were brought to the laboratory. The 1953 sapwood was collected with a drawshave and, instead of immersing the material immediately in absolute methanol, the shavings were air dried; the

assumption being made that the major wood forming enzyme systems were not present in the 1953 sapwood.

The bark was removed easily from the aspen logs during the first three field trips. On August 16, however, the bark was difficult to remove. This was taken as an indication of the termination of cambial activity in this species. Another indication that the growing season was drawing to a close was the appearance of red leaves on some maple trees near the collection site.

Figures 1 and 2 are cross-section reproductions showing the cambium of aspen (in about the middle of each photomicrograph) in the dormant and active states, respectively. The sample for Figure 1 was obtained on February 19, 1954; the sample for Figure 2 on July 13, 1954. It can be seen that the cambium, and the wood and bark elements as well, expand considerably with the resumption of growth in the spring of the year.

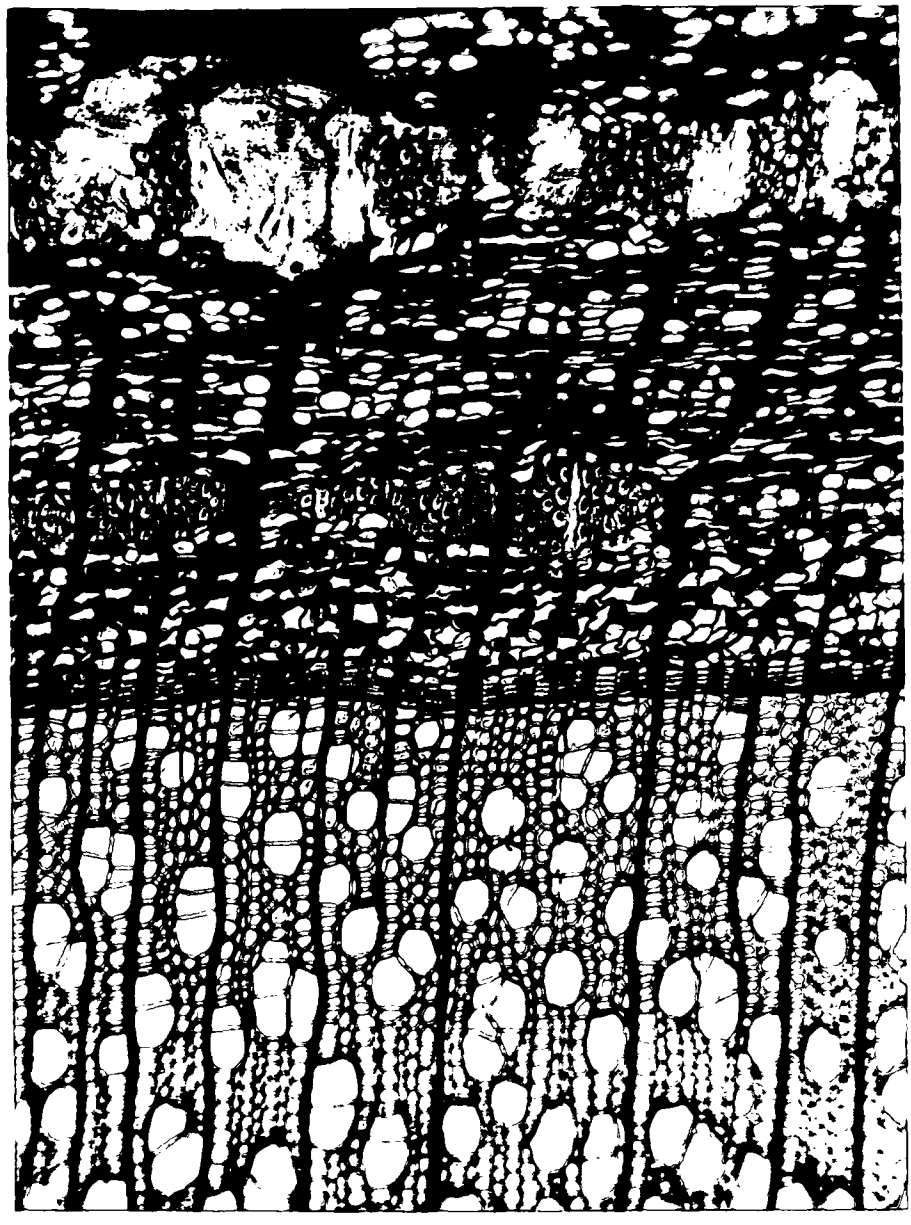

Figure 1

Cross Section Showing Dormant Aspen Cambium, 140X

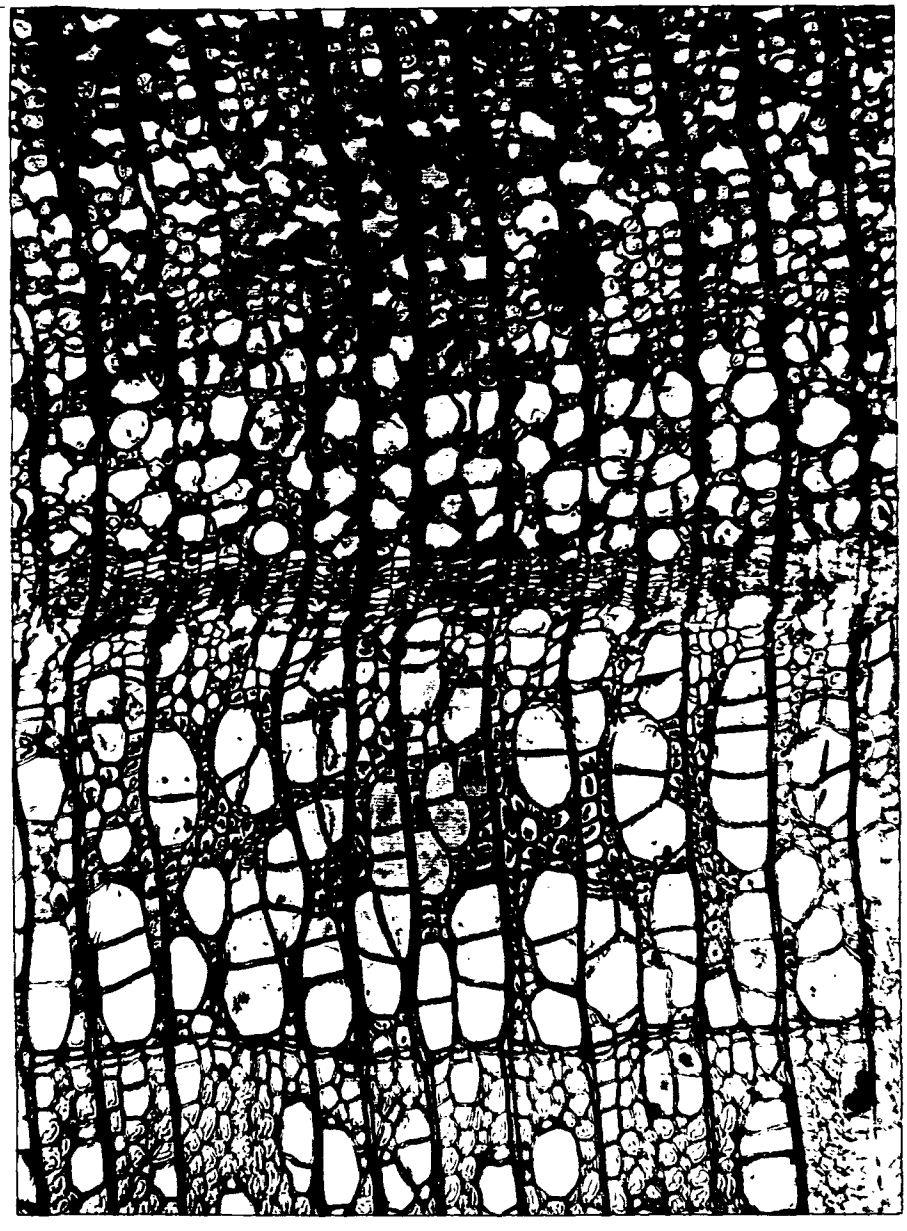

Figure 2

Cross Section Showing Active Aspen Cambium, 140X

MOISTURE CONTENT OF FRESH TISSUES

On the first field trip the method used for moisture determinations required a fixed volume of methanol. The method failed because of rapid evaporation of the methanol during collection of the tissues. On subsequent trips the Karl Fischer method (11) was used to avoid this difficulty. The moisture content of the fresh tissue was calculated from the equation,

$$M = 100H/(S + T + H),$$

where

M = moisture content of the fresh tissue;
H = weight of water in the tissue as determined by the Karl Fischer method;
S = weight of solids extracted by the methanol solvent; and
T = weight of the ovendry tissue.

The procedure was as follows: Small representative samples of SX and 54 tissues were immersed in absolute methanol in ground-glass, stoppered flasks immediately after being separated from the billets. A blank of the same volume was decanted into another flask so that a correction could be made for the small amount of water vapor absorbed.

After the samples had been brought to the laboratory, the methanol solutions were decanted from the various samples and the volumes were measured. Karl Fischer titrations were made on aliquots from each extract. The tissues were recovered with absolute methanol and, after the second extraction had continued at room temperature for 24 hours, the solutions were decanted and aliquots titrated as

before. The amount of water removed in the second extraction was so small that a third extraction was deemed unnecessary. Analogous titrations on the blanks gave corrections for absorbed moisture.

A second aliquot from each extract was evaporated to dryness in a vacuum desiccator to determine the amount of dissolved solids. The extracted tissues were then oven dried and weighed. The results are given in Table II. Each value represents a single determination.

TABLE II

MOISTURE CONTENT OF SOME YOUNG ASPEN TISSUES[1]

| Tissue | Moisture Content, % | Tissue | Moisture Content, % |
|---|---|---|---|
| SX-2 | 90.1[2] | 54-2 | 69.7 |
| SX-3 | 83.0 | 54-3 | 64.3 |
| SX-4 | 70.5 | 54-4 | 57.4 |

[1]All calculations were made on the basis of the weight of the fresh tissues.

[2]Actual moisture content in the tree may be somewhat higher than these values because of loss in obtaining samples.

The table illustrates strikingly the extremely succulent nature of the SX tissue. As expected, the SX tissue had a higher moisture content than the more mature 54 tissue. The high moisture content of the SX tissue was not surprising in view of the important role that water plays in metabolism. It not only serves as the solvent or dispersant for all constituents of protoplasm, but it

also serves as the vehicle by which products of photosynthesis and mineral constituents are brought to the cambial cells and by which metabolic products are carried away.

The moisture content of the 54 tissue may be looked upon as a balance between two factors. On the one hand, as the cells mature there is a decrease in moisture content resulting from the displacement of water by the deposition of secondary cell-wall components and a loss in the capacity of the protoplasm to hold water. On the other hand, an increase in moisture content occurs when the fully developed vessel cells assume their role in the water transportation system of the tree.

COLLECTION OF XYLEM SCRAPINGS

Since qualitative and quantitative tests showed that the SX tissue contained some lignin, a decision was made to attempt to collect a small amount of very young, nonlignified xylem tissue. Accordingly, on June 1, 1955, a trip was made to Eagle River and a tree was selected about 1/4 mile from the site of the previous collections. The tree was felled and sectioned; the bark was removed and discarded; and the very youngest xylem tissue was removed with extreme care. The material was scraped lightly from each billet by holding the knife blade perpendicular to the log surface rather than almost parallel to it as in the collection of the SX tissue. In this way a colloidal mass containing some fibrous material was removed from the log surface. This material was called xylem scrapings, "XSc", and was immersed immediately in

absolute methanol in the usual manner. A Wiesner test carried out in the field indicated that the xylem scrapings were not lignified. Microscopic examination showed that the material contained only xylem elements.

Figure 3 is a phase microscope reproduction of a cross section of the XSc tissue. The shriveled cambial cells can be distinguished just above the thin-walled xylem derivatives. This particular fragment is not truly representative of the whole XSc tissue. It is one of the more mature fragments and was used because the very thin-walled cambial material was virtually impossible to section.

Figure 4 is a photomicrograph of an XSc fragment taken after the tissue had been extracted, air dried, and ground to pass a 40- mesh screen. It can be seen that the material was very thin and exhibited very little of the cell-wall structure that would be evident in a similar fragment from mature wood tissue.

It should be pointed out here that the Molisch, ferric chloride, Mäule, and Wiesner tests were used to indicate the presence or absence of materials of interest in this study. Positve Molisch and ferric chloride tests indicate the presence of carbohydrates and phenols, respectively. The Mäule and Wiesner tests indicate the presence or absence of lignin or materials that might be related to lignin. It is thought that a positive Mäule test indicates the presence of materials having a modified 1, 2, 3-trihydroxybenzene type of structure (12) and that a positive Wiesner test indicates the presence of materials containing the coniferaldehyde group (13). These and other qualitative tests used in this investigation are described in Appendix II, page 111.

Figure 3

Cross-Section of XSc Tissue, 250X

Figure 4

A Typical XSc Fragment, 300X
(Note lack of structural detail.)

## PREPARATION OF MATERIAL

### EXTRACTION OF TISSUES

The SX, 54, and 53 tissues were worked up as shown schematically by the flowsheet on the following page and as described below.

The tissues were extracted with 90% methanol at room temperature for 16 days. Preliminary experiments had indicated that four successive four-day extractions with intermittent agitation was ample time for complete extraction with this solvent. The 53-3 and 53-4 tissues which had been removed in the laboratory rather than at the collection site were air dried, ground in a Wiley mill to pass a 10-mesh screen, and extracted for the full 16 days with 90% methanol. The other tissues were extracted for the first four days with the absolute methanol in which they had been immersed originally, and thereafter with 90% methanol. After each four-day extraction period, the extract was decanted through an 80-mesh screen and the tissues were washed with absolute methanol. The washings were then decanted through the wire screen and combined with the extract. The wire mesh served to retain tissue fragments that otherwise might have contaminated the extract.

Since the 53-3 and 53-4 tissues had been ground prior to extraction, much fine fiber debris passed through the 80-mesh screen. Consequently, the extracts from these tissues were decanted through a medium porosity sintered-glass filter funnel. The first four-day methanol extract from 54-3 and 54-4 contained

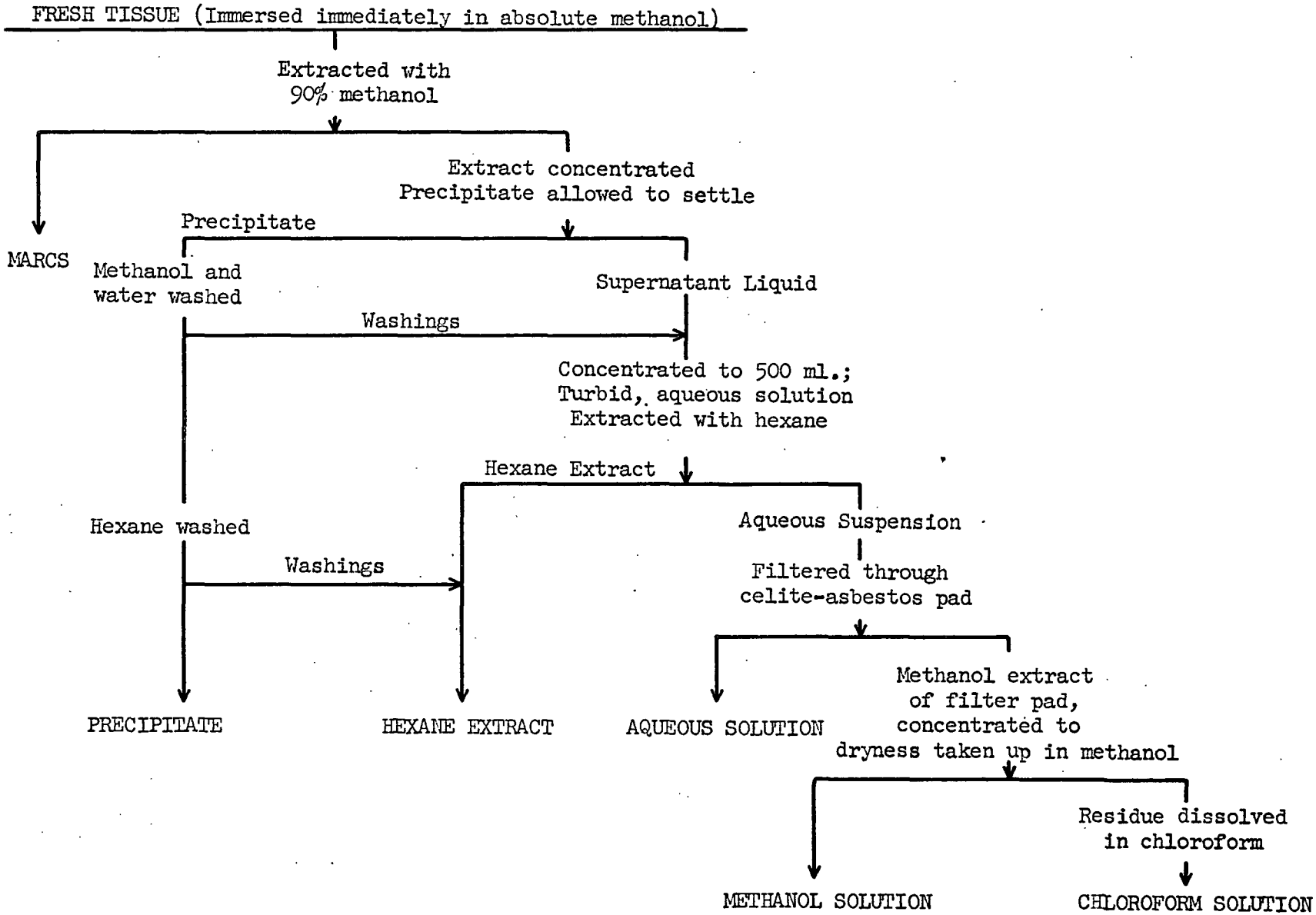


Figure 5

Flowsheet Showing Preparation of SX, 54, and 53 Extracts

suspended, nonfibrous material, but subsequent extracts appeared to contain only finely divided fibers, so the latter were also decanted through the sintered-glass filter funnel.

The extracts in general had a yellow color, the SX extracts being the most intense and the 53 extracts being the least intense. The intensity of the yellow color, of course, diminished with continued extraction.

After the tissues from the first collection period had been extracted with methanol at room temperature for 16 days, they were extracted with absolute methanol under reflux for four hours. No additional material could be extracted in this way. Subsequently the tissues were extracted with water at room temperature for three days. The cold water extracted 0.9, 0.6, and 0.5 gram of material from 15 grams of SX-1 tissue, 35 grams of 54-1 tissue, and 92 grams of 53-1 tissue, respectively. The pH of the SX-water extract was 6.5; that of the distilled water used was 6.8. All the water extracts gave negative Maüle and ferric chloride tests. The Molisch tests were only faintly positive. A glucose solution was made up to the concentration corresponding to the concentration of water-soluble materials indicated in the soft xylem extract. The glucose solution gave a very stongly positive Molisch test. On the basis of the qualitative tests, it was decided not to make a water extraction of the tissues.

After the tissues had been extracted, they were air dried in the hood and ground in a Wiley mill to pass a 40-mesh screen.

Since the XSc extract was not to be investigated, a modified procedure was used. The methanol in which the XSc tissue had been immersed during collection was decanted through an 80-mesh screen which retained the fibrous material. A small amount of nonfibrous, methanol-insoluble material passed through the screen and was removed from the extract by centrifugation. It gave negative Molisch, Mäule, and Wiesner tests, but a positive Biuret test for proteins. Accordingly, the insoluble material was not investigated further.

The fibrous material was extracted twice for one-hour periods with 90% methanol under reflux, air dried, and ground to pass a 40-mesh screen. The material, when dry, was horny and brittle and weighed 2.5 grams; it gave negative Mäule and Wiesner tests.

CONCENTRATION OF EXTRACTS

The methanol extracts of the tissues from the first field trip were concentrated batchwise under vacuum at a temperature not exceeding 35°C. Thereafter, however, a continuously circulating vacuum concentrator was used, resulting in a considerable saving of time. The temperature of the extract was kept below 35°C. at all times.

Invariably, the extracts had a cloudy, opalescent appearance. Removal of the insoluble material from the original extracts was very difficult and time consuming. However, after the extracts had been partially concentrated, the insoluble material settled out on standing. Therefore, the extracts were concentrated to a

volume of two liters in a large concentrator; the concentrator was washed out with two liters of absolute methanol; the concentrate and washings were combined; and the insoluble material was allowed to settle. The supernatant liquid was siphoned off and concentrated to 250 ml. in a small concentrator. The concentrator was washed out with 250 ml. of absolute methanol; the concentrate and washings were combined; and the insoluble material was again allowed to settle. It was necessary to separate the insoluble material in two steps. Otherwise it accumulated on the inside of the concentrator where, because of its general insolubility, it could be removed only with the greatest difficulty.

The supernatant liquid from the second concentrate was siphoned off, and the two precipitates from each extract were combined. These precipitates were washed with water until the washings gave a negative Molisch test. They were then washed three times with absolute methanol, twice with hexane, and dried under vacuum at room temperature. Molisch, Mäule, and Wiesner tests and yield determinations were made on all the precipitates. The results are presented in Table XXII, Appendix I, page 109 . The precipitates were kept, but were not examined further.

The condensates obtained during the concentration of the various extracts were tested qualitatively for carbohydrates and phenols to check for losses due to bumping. In all cases the ferric chloride, Mäule, and Molisch tests were negative, so the condensates were discarded.

The supernatant liquid from the second concentrate was combined with the water and methanol washings and the mixture was again concentrated to 250 ml. in the small circulating concentrator. In order to be reasonably certain that all the methanol had been driven off, three successive 250-ml. portions of distilled water were added and the extract was concentrated finally to 250 ml. A very turbid concentrate was obtained. The concentrator was then rinsed with an additional 250 ml. of distilled water and the rinsings and concentrate were combined to give a final volume of 500 ml.

SEPARATION OF CONCENTRATED EXTRACTS

The concentrated methanol extracts were separated into hexane-, water-, methanol-, and chloroform-soluble fractions as described below.

The turbid, aqueous concentrates were extracted twice with 200 ml. of hexane to remove fats and related substances. The first hexane extracts had a pale yellow-green color. The hexane extracts were then combined with the precipitate washings and concentrated to 500 ml. The hexane solutions gave negative ferric chloride, Molisch, and Wiesner tests. However, when a few drops of these solutions were spotted on filter paper and sprayed with a solution of ferric ferricyanide, phenols were indicated. The hexane solutions were set aside in the cold room, but were not examined further.

The aqueous concentrates were still turbid after hexane extract-

ion. Preliminary experiments indicated that the materials causing the turbidity could be removed by filtering through a celite-asbestos pad, and that this material could then be extracted from the pad with absolute methanol.

The pad was formed by coating the surface of a coarse, sintered-glass filter funnel with medium-length, acid-washed asbestos followed by a thin precoat of analytical grade celite. Both materials were washed with methanol and water prior to use. This type of pad was found to give the fastest filtration.

A mixture of medium-length, washed asbestos (20-25%) and washed analytical grade celite (75-80%) was added to the turbid, aqueous concentrate until the mixture became noticeably thick. The thickened mixture was then poured onto the coated, sintered-glass filter funnel. After the mixture had been filtered, the semidry pad was removed from the filter funnel and mixed with distilled water in a beaker. Again the slurry was filtered through the coated, sintered-glass filter funnel and the pad was washed with water until the filtrate gave a negative Molisch test. Reslurrying the celite-asbestos pad gave faster filtration than washing the pad on the filter funnel.

The aqueous filtrate was still somewhat opalescent so it was filtered again, this time through a medium-porosity, sintered-glass filter funnel on which a celite-asbestos precoat had been formed. This second filtrate was clear, and it was concentrated to 500 ml. The concentrate was used for qualitative tests, yield determinations,

and chromatography; and it was then covered with toluene and frozen to prevent compositional changes.

The pads through which the turbid, aqueous solutions had been filtered were combined, in the case of each extract, and mixed thoroughly with absolute methanol in a beaker. The mixture was then filtered through a celite-asbestos coated, medium-porosity, sintered-glass filter funnel. When the pad was dry, it was removed from the filter funnel and reslurried with methanol. The slurry was filtered again through a coated, medium-porosity, sintered-glass filter funnel. The pad was washed with methanol until the filtrate did not become cloudy on the addition of water. The dry pad gave negative Molisch, ferric chloride, Mäule, and Wiesner tests, indicating that the carhohydrates and phenols had been removed.

When an attempt was made to concentrate the methanol solutions to a smaller volume in a circulating concentrator, some material began to separate and the originally clear solutions became turbid and, hence, unsuitable for chromatography. Therefore, after each methanol solution had been concentrated to 500 ml. in the circulating concentrator, the turbid solution was transferred to a one-liter round-bottomed flask and reduced to a sirup under vacuum at a temperature of 20-21°C. This sirup was taken up in a small amount of absolute methanol; the resulting solution was used for qualitative tests, yield determinations, and chromatography, and was kept in a cold room to prevent compositional changes.

Invariably there was some material resembling soft taffy that did not redissolve; it was soluble in chloroform, insoluble in benzene and ethyl acetate, and formed a flocculent precipitate in ether and hexane. The material was taken up in a small amount of chloroform. The chloroform solutions gave negative ferric chloride, Molisch, and Wiesner tests. However, when a few drops of the solutions were spotted on filter paper and sprayed with a solution of ferric ferricyanide, phenols were indicated. The chloroform solutions were set aside in the cold room, but were not examined further.

## YIELDS AND QUALITATIVE TEST RESULTS

Table III shows the amount of water-, hexane-, and methanol-soluble material present in each extract. The relatively small amounts of precipitated and chloroform-soluble material (Figure 5, page 25) are not included. A value for the total solids extracted with 90% methanol was obtained by adding together the values for the three fractions. For yield determinations, aliquots of the methanol and hexane solutions were evaporated to dryness in a vacuum desiccator at room temperature. Because severe bumping occurred when a similar procedure was followed in the case of aqueous aliquots, it was necessary to concentrate the solutions to a sirup on a steam bath prior to drying in a vacuum desiccator.

TABLE III

YIELDS OF WATER-, METHANOL-, AND HEXANE-SOLUBLE MATERIALS EXTRACTED FROM ASPEN TISSUES WITH 90% METHANOL[1,2]

| | SX-1 | SX-2 | SX-3 | SX-4 | 54-1 | 54-2 | 54-3 | 54-4 | 53-1 | 53-2 | 53-3 | 53-4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| In water, g. | 13.48 | 40.81 | 75.31 | 7.18 | 3.26 | 21.06 | 28.79 | 52.76 | 0.81 | 0.77 | 8.47 | 3.22 |
| In methanol, g. | 1.06 | 2.54 | 5.34 | 0.32 | 0.61 | 1.86 | 6.32 | 7.26 | 1.32 | 0.59 | 17.00 | 11.59 |
| In hexane, g. | 0.55 | 1.21 | 1.59 | 0.64 | 0.34 | 1.54 | 1.95 | 2.45 | 0.54 | 0.44 | 1.69 | 0.93 |
| Total extractives, g. | 15.09 | 44.56 | 82.24 | 8.14 | 4.21 | 24.46 | 37.06 | 62.47 | 2.67 | 1.80 | 27.16 | 15.74 |
| Weight of marc, g. | 15.1 | 39.4 | 138 | 10.2 | 34.6 | 176 | 475 | 534 | 91.8 | 47.5 | 808 | 468 |
| Total weight, g. | 30.2 | 84.0 | 220 | 18.3 | 38.8 | 200 | 512 | 596 | 94.5 | 49.3 | 835 | 484 |
| In water, % | 44.7 | 48.6 | 34.2 | 39.2 | 8.4 | 10.6 | 5.6 | 8.9 | 0.9 | 1.6 | 1.0 | 0.7 |
| In methanol, % | 3.5 | 3.0 | 2.4 | 1.7 | 1.6 | 0.9 | 1.2 | 1.2 | 1.4 | 1.2 | 2.0 | 2.4 |
| In hexane, % | 1.8 | 1.4 | 0.7 | 3.5 | 0.9 | 0.8 | 0.4 | 0.4 | 0.6 | 0.9 | 0.2 | 0.2 |
| Total extractives, % | 50.0 | 53.0 | 37.3 | 44.4 | 10.9 | 12.3 | 7.2 | 10.5 | 2.9 | 3.7 | 3.2 | 3.3 |

[1]Calculations were made on the basis of the ovendry, ash-free, unextracted tissue.

[2]The reader is referred to Figure 5, page 25, for origin of these materials.

Tables IV, V, and VI show the results of some of the qualitative tests run on the water- (W) and methanol- (A) soluble fractions.

TABLE IV

QUALITATIVE TEST RESULTS FOR SX-W AND SX-A FRACTIONS

| Solubility fraction | SX-W | | | | SX-A | | | |
|---|---|---|---|---|---|---|---|---|
| Collection period | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Molisch test | + | + | + | + | + | + | + | + |
| Mäule test | - | + | + | - | - | - | - | - |
| Wiesner test | - | - | - | - | - | - | - | - |

TABLE V

QUALITATIVE TEST RESULTS FOR 54-W AND 54-A FRACTIONS

| Solubility fraction | 54-W | | | | 54-A | | | |
|---|---|---|---|---|---|---|---|---|
| Collection period | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Molisch test | + | + | + | + | + | + | + | + |
| Mäule test | - | + | + | + | - | + | + | + |
| Wiesner test | - | + | + | + | - | - | + | + |

TABLE VI

QUALITATIVE TEST RESULTS FOR 53-W AND 53-A FRACTIONS

| Solubility fraction | 53-W | | | | 53-A | | | |
|---|---|---|---|---|---|---|---|---|
| Collection period | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Molish test | + | + | + | + | - | + | - | - |
| Maüle test | - | - | + | + | - | - | + | + |
| Wiesner test | - | - | + | + | - | - | + | + |

It is evident from the tables that nearly all of the aqueous and methanol fractions contained carbohydrates and that Maüle- and Wiesner-positive substances were sparse in the SX, and early 54 and 53 fractions. The presence of Maüle- and Wiesner-positive materials in the 54-W fractions may indicate the presence of low molecular weight lignin precursors because lignification should have been strongest in the 54 tissues.

In addition to the tests listed in the tables, the ferric chloride test indicative of phenols the Isenberg-Buchanan test, said to indicate the presence of catechol tannins (14) and the naphthoresorcinol test for uronic acids were run on the aqueous and methanol solutions. None of these tests were positive. The failure of the ferric chloride test could have been due to the presence of organic acids, which are known to interfere. A negative naphthoresorcinol test cannot be taken as proof of the absence of uronic acids because aldobiuronic acids sometimes do not respond (15).

SUMMARY OF PREPARATION OF MATERIALS

The following materials were prepared for analysis. The SX, 54, and 53 tissues from each of the four field trips were extracted with 90% methanol leaving as the insoluble residue the 12 marcs SX-1, SX-2, SX-3, SX-4; 54-1, 54-2, 54-3, 54-4; and 53-1, 53-2, 53-3, 53-4. One XSc marc was also prepared. Each of the 12 extracts was concentrated and separated into a precipitate and fractions soluble in hexane, chloroform, water, and methanol. Qualitative tests showed that the water and methanol solutions corresponding to the 12 marcs contained the soluble materials most relevant to this investigation.

# ANALYSIS OF MARCS

## OVENDRY WEIGHT AND ASH DETERMINATIONS

Ovendry weight (16) and ash (17) assays were made on all marcs. It was found necessary to keep the samples in an oven at 105°C. for as long as 24 hours before they reached constant weight.

All calculations in the study of marcs were made on an ovendry, ash-free, extractive-free basis unless otherwise specified.

## NITROGEN DETERMINATION

Nitrogen determinations were made on all marcs by a modified Kjeldahl method (18); the results are presented in Table VII. The protein content was obtained by multiplying the nitrogen content by 6.25.

TABLE VII

SUMMARY OF NITROGEN ANALYSES[1]

| Tissue | Nitrogen, % | Protein, % |
|---|---|---|
| XSc | 4.4[2] | 27.3 |
| SX-1 | 1.6 | 10.0 |
| SX-2 | 1.9 | 11.9 |
| SX-3 | 1.1 | 6.9 |
| SX-4 | 1.0 | 6.3 |
| 54-1 | 0.4 | 2.5 |
| 54-2 | 0.4 | 2.5 |
| 54-3 | 0.2 | 1.3 |
| 54-4 | 0.1 | 0.6 |
| 53-1 | 0.1 | 0.6 |
| 53-2 | 0.1 | 0.6 |
| 53-3 | 0.1 | 0.6 |
| 53-4 | 0.1 | 0.6 |
| Whole wood[3] | 0.1 | 0.6 |

[1]All calculations were made on the basis of the ovendry, ash-free marcs.

[2]This determination was carried out by the Analytical Department of The Institute of Paper Chemistry—Institute Method 708.

[3]The whole wood sample used in this and subsequent analyses was obtained from 3 billets representing 3 different aspen trees, from which the developing tissues had been removed.

The table shows that the XSc and SX marcs are very rich in proteins. This was to be expected in view of the fact that these tissues contain the protoplasm from which the cell constituents are derived. Obviously, the 54 marcs represent an intermediate growth stage. The protein content of the 53 marcs is the same as that of the whole wood.

## URONIC ANHYDRIDE DETERMINATION

Uronic anhydride assays (19) were made on all marcs. The apparatus, reagents, and technique were checked with pure glucuronolactone. The results of these analyses are presented in Table VIII.

TABLE VIII

SUMMARY OF URONIC ANHYDRIDE ANALYSES[1]

| Tissue | Uronic Anhydride, % |
|---|---|
| XSc | 23.0[2] |
| SX-1 | 12.5 |
| SX-2 | 11.7 |
| SX-3 | 8.6 |
| SX-4 | 9.6 |
| 54-1 | 6.5 |
| 54-2 | 6.1 |
| 54-3 | 5.5 |
| 54-4 | 5.7 |
| 53-1 | 5.5 |
| 53-2 | 5.1 |
| 53-3 | 5.0 |
| 53-4 | 5.2 |
| Whole wood | 5.6 |

[1]All calculations were made on the basis of the ovendry, ash-free marcs.

[2]This determination was carried out by the Analytical Department of The Institute of Paper Chemistry--Institute Method 701.

This table shows that the young XSc and SX tissue are characterized by a high uronic anhydride content and the mature 53 tissue by a low uronic anhydride content; the 54 tissue again seems to represent an intermediate growth stage. The uronic anhydride content of the 53 tissue is nearly the same as that of the whole wood. It is evident that as the young tissue matures the relative amount of carbohydrate material containing carboxyl groups decreases markedly.

## METHOXYL DETERMINATION

Methoxyl determinations (20) were made on all marcs. The apparatus, reagents, and technique were checked with pure vanillin.

Since the marcs had been air dried from methanol, it was necessary to remove the last traces of alcohol before making the determinations. This was accomplished by exposing the airdry marcs to a high relative humidity and then drying the moist marcs in a vacuum desiccator over sodium hydroxide. The procedure was repeated four times.

The results of the methoxyl determinations are presented in Table IX.

TABLE IX

SUMMARY OF METHOXYL DETERMINATIONS[1]

| Tissue | Methoxyl, % |
|---|---|
| XSc | 2.2 |
| SX-1 | 2.6 |
| SX-2 | 2.7 |
| SX-3 | 3.4 |
| SX-4 | 3.2 |
| 54-1 | 4.9 |
| 54-2 | 4.2 |
| 54-3 | 5.2 |
| 54-4 | 5.6 |
| 53-1 | 6.0 |
| 53-2 | 5.6 |
| 53-3 | 5.6 |
| 53-4 | 6.2 |
| Whole wood | 5.9 |

[1]All calculations were made on the basis of the ovendry, ash-free marcs.

The table shows that the methoxyl contents go from a low value in the case of the XSc marc to a high value in the 53 marcs and whole wood, with the 54 marcs being intermediate. The methoxyl in the XSc marc must be carbohydrate methoxyl since, as shown below, this material gives no vanillin or syringaldehyde on oxidation with alkaline nitrobenzene. The increase in methoxyl occurring after the XSc growth stage is due primarily to the deposition of lignin, although some methoxyl associated with hemicelluloses is undoubtedly deposited also.

## LIGNIN DETERMINATION

It is known that proteins and some carbohydrates condense with lignin when the Klason method is used. This is particularly likely in the case of cambial tissues which have a very high protein content. Therefore, the lignin content of the marcs was calculated from two sets of data. One calculation was based on the yields of vanillin and syringaldehyde obtained by oxidation of the marcs with alkaline nitrobenzene; the other calculation was based on the methoxyl and uronic anhydride contents of the marcs. In order to make these calculations, the lignin content of whole aspenwood was required. This assay was made by the Klason method (21). The soluble lignin in the filtrate was determined spectrophotometrically from the relation,

$$C_L = A/ab,$$

where

$C_L$ = concentration of lignin in grams/liter;
A = absorbance;
a = absorptivity; and
b = cell width in cm.

The value of "a" at 230 mmu is 42 according to the ultraviolet absorption data for aspen native lignin (22). The soluble lignin was added to the Klason lignin to give a total lignin value.

### FROM NITROBENZENE OXIDATION

The micromethod (23) for the alkaline nitrobenzene oxidation of lignin and determination of aldehydes, as modified for aspenwood (24) was used. The lignin content was calculated from the relation,

$$L_n = K(a + b)/(x + y), \qquad (25)$$

where

$L_n$ = lignin content at any growth stage;
K = Klason lignin content of the whole wood;
a,b = yield of vanillin and syringaldehyde from the tissue being investigated; and
x,y = yield of vanillin and syringaldehyde from the whole wood.

Calculating the lignin content of young tissues in this way involves two assumptions: (a) that the vanillin and syringaldehyde produced arise solely from the lignin, and (b) that vanillin and syringaldehyde make up the same fraction of the lignin in both young and mature tissues.

FROM METHOXYL AND URONIC ANHYDRIDE VALUES

The total methoxyl content of a given wood sample is the sum of the lignin methoxyl and the uronic anhydride methoxyl. Hence, if the total methoxyl and uronic anhydride methoxyl of a given sample are known, the lignin methoxyl can be determined by difference. The lignin content can then be estimated from the relation,

$$L_m = K(c/d),$$

where

$L_m$ = lignin content at any growth stage;
K = Klason lignin content of the whole wood;
c = lignin methoxyl of the tissue being investigated; and
d = lignin methoxyl of the whole wood.

Use of this relation assumes that the methoxyl content of young and mature lignin is the same.

The total uronic anhydride content of a given marc is the sum of the galacturonic and glucuronic anhydrides. The former is a constituent of the pectic substances and the latter a constituent of the nonpectic hemicelluloses. Each fraction probably has a different methoxyl content. Therefore, in order to calculate the uronic anhydride methoxyl, the galacturonic and glucuronic anhydride content of each marc and the methoxyl content of the respective anhydrides must be estimated.

If it is assumed that the XSc uronic anhydride arises solely from galacturonic anhydride (see page 52, 53) and that the pectic substances are deposited completely and by the XSc growth stage (see page 58), the magnitude of each anhydride can be determined. Table XV, page 61 shows that galacturonic anhydride accounts for about two-thirds of the total uronic anhydride in SX tissue and about one-third of the total uronic anhydride in the 54 and 53 tissues. Furthermore, the methoxyl content of the galacturonic anhydride fraction is known from assays of the XSc marc, and the methoxyl content of the glucuronic anhydride fraction can be assumed to be approximately equivalent to one methoxyl group per glucuronic anhydride group.

The calculations involved are shown in Appendix III, page 114.

RESULTS AND DISCUSSION

The results of these determinations are given in Table X. It can be seen that both methods give very similar results.

TABLE X

CALCULATED YIELDS AND QUALITATIVE STUDIES OF LIGNIN IN YOUNG ASPEN TISSUES[1]

| Tissue | Lignin From Nitrobenzene Oxidations, % | Lignin From Methoxyl Determinations, % | Maüle Test | Wiesner Test |
|---|---|---|---|---|
| XSc | 0.0 | 0.0 | - | - |
| SX-1 | 5.2 | 4.5 | + | + |
| SX-2 | 5.1 | 5.3 | + | + |
| SX-3 | 7.3 | 9.4 | ++ | ++ |
| SX-4 | 7.9 | 8.2 | ++ | ++ |
| 54-1 | 15.8 | 15.9 | ++++ | ++++ |
| 54-2 | 14.4 | 13.5 | ++++ | ++++ |
| 54-3 | 17.5 | 17.9 | ++++ | ++++ |
| 54-4 | 18.6 | 19.2 | ++++ | ++++ |
| 53-1 | 22.4 | 21.2 | ++++ | ++++ |
| 53-2 | 20.7 | 19.6 | ++++ | ++++ |
| 53-3 | 21.0 | 20.0 | ++++ | ++++ |
| 53-4 | 20.9 | 22.0 | ++++ | ++++ |
| Whole wood (Klason + soluble lignin) | 20.8 | 20.8 | ++++ | ++++ |

[1]All calculations were made on the basis of the ovendry, ashfree marcs.

Both the qualitative and quantitative data indicate that there are no completely lignified cells in the SX tissue. The 54 tissue must be composed of a mixture of partially lignified and completely lignified cells. There is no doubt that the partially lignified cells were still functioning as living cells at the time of collection. Whether or not the completely lignified vessel segments and fiber tracheids were still living is a moot question. The cells may live, in the sense that they are capable of respiration, for some time after cell-wall formation has been completed.

In this study, the ratio of syringaldehyde to vanillin was variable (average value = 2.7), but did not seem to change significantly as the lignin matured. In contrast, investigations of development of lignin in monocotyledonous plants (25, 26) have shown that the young lignins have a much lower syringaldehyde to vanillin ratio and, consequently, a lower methoxyl content (27) than mature lignins.

The only aldehyde found upon oxidation of the XSc marc with alkaline nitrobenzene was p-hydroxybenzaldehyde of which there was a very small amount. Since this aldehyde could not be detected among the oxidation products of the other tissues, it was probably produced from the proteins in the XSc marc. It is known that tyrosine yields p-hydroxybenzaldehyde upon oxidation with alkaline nitrobenzene (25). No syringaldehyde or vanillin could be detected. Hence the XSc marc was not lignified.

## DETERMINATION OF CELL-WALL CARBOHYDRATES

The polysaccharides in the marcs were hydrolyzed completely to simple sugars in two stages to minimize decomposition of the end products (28). After adding a known amount of D-ribose (about equal to the amount of glucose in the hydrolyzate) as an internal reference sugar, the hydrolyzate was taken to pH 4.5 with barium hydroxide, filtered, deionized, and concentrated *in vacuo* (29).

A volume of the hydrolyzate equivalent to 8-10 mg. of sugar was then carefully streaked across the top of a sheet of Whatman No. 1 chromatographic paper and the sugar mixture resolved by alternate

developing and drying in EAW (9:2:2) and BPW (10:3:3) (29). It was found that a third 12-hour development in BPW (10:3:3) in addition to the two suggested, gave a somewhat better separation of glucose and galactose.

After the chromatogram had dried, a strip was cut from each side and sprayed with AHP. The strips were then reattached to locate and identify the individual sugar bands. The bands and a blank were cut from the chromatogram and the sugars eluted with water (29). About 2 1/2 hours was required for complete elution. Although the alternate developing and drying method resulted in more compressed and more sharply separated sugar bands, the quantity of glucose, xylose, and ribose was such that wide bands were often obtained. These wider bands could not be eluted completely in 2 1/2 hours, so they were cut in two, each half was eluted separately, and the two eluates were combined.

The periodate oxidation method (30) was used for the quantitative determination of the eluted sugars because it gives good results with hydrolyzed aspenwood (29).

The weight of the various sugar anhydrides was calculated from the relation,

mg. sugar anhydride = ABC,

where

A = mg. ribose added/(NaOH titer for ribose X pentose factor);
B = NaOH titer X sugar factor; and
C = sugar anhydride factor.

The sugar factors are necessary because hexoses, pentoses, and methyl pentoses yield 5 moles, 4 moles, and 4 moles of formic acid, respectively, on oxidation with periodate. Thus, one mole of formic acid is equivalent to 1/5 mole or 36.03 grams of a hexose sugar, 1/4 mole or 37.53 grams of a pentose sugar, and 1/4 mole or 41.04 grams of a methyl pentose sugar. The sugar anhydride factor is necessary to convert the weight of the simple sugar to the weight of the corresponding cell wall carbohydrate.

In order to determine the accuracy and precision with which the simple sugars composing the polysaccharides of aspen tissue could be determined, the following experiment was performed. The weighed CP sugars D-glucose, D-galactose, L-arabinose, D-Mannose, D-xylose, L-rhamnose, and the internal reference sugar D-ribose were transferred quantitatively to 10-ml. volumetric flasks and made up to volume with boiled, distilled water. Duplicate 1-ml. aliquots were then oxidized and titrated with .00402 N sodium hydroxide. The precision of the duplicate determinations for all sugars was ±3%; the accuracy is shown in the following table.

TABLE XI

SUMMARY OF QUANTITATIVE SIMPLE SUGAR STUDY

| Sugar | Known, mg. | Found, mg. | Recovery, % |
|---|---|---|---|
| Glucose | 2.92 | 2.80 | 95.5 |
| Galactose | 3.15 | 3.12 | 99.0 |
| Mannose | 2.81 | 2.77 | 98.4 |
| Arabinose | 3.00 | 2.99 | 99.7 |
| Xylose | 3.29 | 3.26 | 99.1 |
| Ribose | 3.09 | 3.13 | 101.2 |
| Rhamnose | 2.96 | 2.93 | 99.0 |

The nonpolyuronide cell-wall carbohydrates were analyzed by hydrolyzing the XSc, SX, 54, and 53 marcs and determining quantitatively the simple sugars of which they were composed. Uronic anhydride assays were carried out as described previously. Table XII summarizes these analyses.

TABLE XII

COMPOSITION AND SEASONAL VARIATION
OF CELL-WALL CARBOHYDRATES[1]

| Tissue | Glucan,[2] % | Xylan,[2] % | Mannan,[2] % | Galactan,[2] % | Araban,[2] % | Methylated Uronic Anhydride,[3] % |
|---|---|---|---|---|---|---|
| XSc | 15.1 | 6.1 | 0.9 | 10.2 | 6.8 | 24.0 |
| SX-1 | 40.6 | 17.1 | 1.3 | 2.6 | 2.3 | 13.2 |
| SX-2 | 41.0 | 16.8 | 0.8 | 3.9 | 2.3 | 12.3 |
| SX-3 | 47.4 | 18.7 | 1.3 | 1.6 | 1.4 | 9.1 |
| SX-4 | 47.2 | 19.9 | 1.0 | 2.2 | 2.1 | 10.2 |
| 54-1 | 48.7 | 18.1 | 1.2 | 0.7 | 0.8 | 7.0 |
| 54-2 | 47.3 | 17.7 | 1.3 | 2.7 | 0.6 | 6.5 |
| 54-3 | 52.2 | 17.9 | 1.8 | 1.1 | 0.6 | 5.9 |
| 54-4 | 45.8 | 15.8 | 1.6 | 0.7 | 0.4 | 6.1 |
| 53-1 | 49.4 | 16.5 | 2.3 | 0.9 | 0.4 | 5.9 |
| 53-2 | 56.7 | 16.3 | 2.1 | 0.7 | 0.4 | 5.5 |
| 53-3 | 51.0 | 16.0 | 2.1 | 0.5 | 0.6 | 5.3 |
| 53-4 | 50.6 | 16.9 | 1.8 | 0.7 | 0.6 | 5.6 |

[1]All calculations were made on the basis of the ovendry, ashfree marcs.

[2]Calculations were made on the basis of the sugar anhydrides; no implication as to structure is intended.

[3]Includes "uronic anhydride $-CH_2-$"--see Appendix IV, page 116.

In addition to the carbohydrates listed in Table XII, rhamnose was also present in small quantities (less than 1%) in all the hydrolyzates. Traces of fucose and ribose were indicated in the XSc hydrolyzate. Neither of these sugars were present in detectable quantities in the hydrolyzates of the more mature marcs. The presence of fucose in the hydrolyzate of a cold water extract of aspenwood had been reported previously (29). However, the identity of this sugar has not been established; it has been indicated only by paper chromatography.

The presence of small quantities of ribose in the hydrolyzate of the XSc marc is not surprising in view of the high protein content of this very young material (see Table VII). Since ribose was not present in the hydrolyzates of the more mature marcs, it probably originated from the nucleic acids in the cambial protoplasm rather than from the cambial cell wall. The XSc carbohydrate analysis probably includes minute amounts of other protoplasmic carbohydrates as well.

THE XSc CELL-WALL CARBOHYDRATES

The XSc tissue was extremely interesting. Microscopic examination showed that it was composed predominantly of cambial cells and also contained some thin-walled xylem elements derived from the cambium. Although the walls of some of the derivative cells were thicker than those of the initials, the analytical results indicated that this thickening was due to growth of the primary wall rather than to secondary-wall formation. Since the cambial cell wall becomes the primary wall of a mature wood cell, the carbohydrates of the XSc tissue correspond to the carbohydrates in the compound middle lamella of mature wood.

This tissue gave negative Mäule and Wiesner tests, and yielded neither vanillin nor syringaldehyde upon oxidation with alkaline nitrobenzene. It was, therefore, nonlignified. Evidently the commencement of lignification in aspen is coincident with commencement of secondary-wall formation.

Although microscopic examination showed that little or no secondary-wall formation had taken place at this growth stage, every sugar present in the hydrolyzate of mature aspenwood was also present in the hydrolyzate of the XSc tissue. Surprisingly, less than 25% of the total carbohydrate material was "glucan".

In view of the relatively high percentage of galactose and arabinose indicated in the XSc tissue, it was deemed advisable to verify the presence of these sugars by other than chromatographic means. Therefore, they were separated chromatographically, eluted, and reduced to a sirup in vacuo. The mucic acid derivative of galactose and the diphenylhydrazone derivative of arabinose were prepared. The former should melt at 206°C. with very slow heating (found: 204°C.) and the latter at 204-205°C (31) (found: 201°C.).

It is known that cambial and near-cambial tissues are rich in pectic substances (2, 4). Furthermore, the XSc tissue gave a strongly positive ruthenium red test --a good indication of the presence of pectic substances. This implies that the polyuronide content of the XSc tissue is primarily polygalacturonic acid. It is also known that, in general, other polysaccharides, especially galactan and araban, are associated with polygalacturonic acid in pectic substances (32). Now, if araban and galactan are included in the term, "pectic substances", and if the XSc uronic anhydride is assumed to be galacturonic anhydride, two-thirds of the total XSc carbohydrate consists of pectic substances. Hence, an outstanding characteristic of the XSc tissue is its predominantly pectic

nature. The very high concentration of these materials together with the very low concentration of "glucan" in the XSc tissue lends support to the hypothesis that the pectic substances (rather than cellulose) represent the continuous phase in the structure of the primary cell wall (33, 34).

The pectic substances have the following properties which make them well suited to the role they play in cell growth: (a) they are highly hydrophilic thus enabling young cells to maintain a high state of hydration, (b) they are readily susceptible to enzymatic hydrolysis thus providing for expansion of the primary wall, (c) under certain conditions they form gels very easily thus making the compound middle lamella adaptable to changes in size and shape of the cell, and under other conditions they can function as cementing substances thus lending rigidity to the young tissue.

It is interesting that the three major carbohydrates of the compound middle lamella (galactose, arabinose, and galacturonic acid) are D-galactose homomorphs and that the three major carbohydrates of the secondary wall (glucose, xylose, and glucuronic acid) are D-glucose homomorphs. Evidently, metabolic conditions prevailing at the time of the formation of the compound middle lamella result in the conversion of the glucose, present in cambial sap, primarily into galactose, arabinose, and galacturonic acid units; whereas during secondary-wall formation, conditions are such that glucose, xylose, and glucuronic acid units predominate.

## SUMMARY OF MARC ANALYSES

The individual analytical values for the various marcs were averaged and are presented in Tables XIII and XIV. Table XIII shows the variation in carbohydrate composition according to tissue type; and Table XIV shows the proximate summative analysis of the various marcs.

In general, the XSc and SX tissues of aspen were characterized by a lower glucan, mannan, lignin, and methoxyl content, and a higher uronic anhydride, galactan, araban, and protein content than mature aspen tissue. The composition of the XSc tissue evidently approaches that of the cambium.

All the 53 values except those for glucan and mannan agree quite well with the values obtained for the whole wood. On the basis of this comparison, the 53 values may be considered typical of the wood as a whole.

TABLE XIII

VARIATION IN CARBOHYDRATE COMPOSITION ACCORDING TO TISSUE TYPE[1]

| Tissue | Glucan, % | Xylan, % | Mannan, % | Galactan, % | Araban, % | Methylated Uronic Anhydride, % | Total Carbohydrate, % |
|---|---|---|---|---|---|---|---|
| XSc | 15.9 | 6.1 | 0.9 | 10.2 | 6.8 | 24.0 | 63.1 |
| SX | 43.8 | 18.1 | 1.1 | 2.5 | 2.0 | 11.2 | 78.7 |
| 54 | 48.5 | 17.5 | 1.5 | 1.4 | 0.6 | 6.4 | 75.9 |
| 53 | 51.6 | 16.4 | 2.1 | 0.7 | 0.5 | 5.6 | 76.9 |
| Whole wood[2] | 41.2 | 15.8 | 1.3 | 0.6 | 0.3 | 6.0 | 65.2 |

[1]All calculations were made on the basis of the ovendry, ash-free marcs.

[2]*cf.* Appendix V, page 117.

TABLE XIV

PROXIMATE SUMMATIVE ANALYSIS OF TISSUES INVESTIGATED[1,2]

| | XSc | SX | 54 | 53 | Whole Wood[3] |
|---|---|---|---|---|---|
| Total carbohydrate, % | 63.1 | 78.7 | 75.9 | 76.9 | 65.2 |
| Lignin, % | 0.0 | 6.4 | 16.6 | 21.3 | 20.8 |
| Protein, % | 27.3 | 8.8 | 1.7 | 0.6 | 0.6 |
| Total, % | 90.4 | 94.0 | 94.2 | 98.8 | 86.6 |

[1]All calculations were made on the basis of the ovendry, ashfree marcs.

[2]No acetyl determinations (usually 3.5-4.0% of whole wood) were made in this study.

[3]cf. Appendix V, page 117.

The 41% whole wood glucan values obtained in this study and in another investigation (29) in which the same method of analysis was used seem low, especially in view of the aspen alpha-cellulose figures of 50.2% (35), 49.1% (36), 50.1% (37), and 50.8% (38) obtained by others. It should be pointed out, however, that the 53 glucan value of 51.6% (Table XIII) is in good agreement with the whole wood alpha-cellulose figures cited above. Nevertheless, the discrepancy remains unexplained.

Although there is little indication of seasonal variation in the polysaccharide composition of the SX and 54 marcs, there does seem to be a seasonal variation in the protein, lignin, methoxyl, and uronic anhydride content. The seasonal variation in the SX marcs may be due to a change in composition of these tissues as the

season progresses. Possibly the best explanation, however, is that the SX-3 and SX-4 tissues contained more of the adjacent newly formed sapwood. This explanation is supported by the observation, made at the time of collection of these tissues, that it was more difficult to make a good separation between the SX and 54 tissues due to a reduction in the moisture content at the log surface.

If, as indicated by the analytical data, the 54 tissue represents an intermediate growth stage, one would expect the composition of the 54 tissue to approach that of the 53 tissue as the season advanced due to the fact that the proportion of fully mature cells in the former increases as the season progresses. This trend is evident in Tables VII, VIII, IX, and X.

## ESTIMATION OF CARBOHYDRATE AND LIGNIN GROWTH INCREMENTS

In order to estimate the quantity of each constituent deposited as the tissue matured through the four growth stages indicated in Table XIII, it would be desirable to have an "internal index", i.e., some constituent which would remain substantially unchanged after being deposited by the first growth stage. Inspection of the table shows that glucan, xylan, and mannan increased and galactan, araban, and uronic anhydride decreased in percentage of composition as the tissue matured. However, since growth is a dynamic process, results which show only the percentage of composition of tissue at various growth stages can be very misleading. For example, a decrease in the percentage of one constituent need not indicate an actual decrease in the absolute quantity of that constituent, but rather an increase in some other constituent or constituents.

The data of Table III suggested the hypothesis that they pectic substances are deposited completely in aspen tissue by the XSc growth stage prior to secondary-wall formation. Evidence in the literature lends support to this hypothesis.

It has been shown that alcohol-soluble uronides are converted into alcohol- and water-insoluble pectic substances in rapidly growing sugar beets (39), in maturing flax (40), and in tobacco pith (41). Apparently the formation of polygalacturonic acid derivatives by the cell is principally an indication of a certain level of oxidative activity met with only in very young cells (42). It has

been suggested that the pectic substances of woody tissues are deposited in the middle lamella and primary wall in the early stages of cell development and that they remain unchanged as the wood matures (43, 44). In fact, they have been identified histologically in the compound middle lamella of mature wood (45) and have been isolated from cambium and mature sapwood tissues (43, 44) after cautious treatment to remove interfering materials.

Thus, the enzymes in developing tissues which are capable of hydrolyzing pectic substances do not bring about the complete break down and subsequent removal of these materials from the compound middle lamella. Indeed, it has been hypothesized that they merely loosen the continuous pectic framework of the primary wall to accommodate its expansion during growth (46). Similarly, there is no evidence for the degradation of pectic substances during the development of fruit. However, when the fruit becomes overripe and approaches senescence there is a gradual decrease in these materials. It may well be that the degradation of pectic substances in wood is prevented by the deposition of lignin in the compound middle lamella.

The constituents of Table XIII which may be considered to represent pectic substances are araban, XSc, uronic anhydride (assumed to be galacturonic anhydride), and galactan (32). Of these, araban was chosen as the best internal index of cell growth because: (a) the uronic anhydride determination does not distinguish between the uronic acids of the pectic and nonpectic substances, and (b) the

quantitative determination of arabinose was more accurate than that of galactose. The separation of arabinose from the monosaccharides above and below it on the chromatogram in the procedure for quantitative sugar determinations was very good, whereas the separation of galactose from the necessarily large quantity of glucose below it on the chromatogram left something to be desired.

The calculations involved in using the internal index concept for estimation of the various carbohydrate and lignin growth increments are shown in Appendix VI, page 118. The values in Table XIII were recalculated on the basis of the ovendry, ash-, and protein-free marcs, and the growth increment calculations were based on 100 grams of ovendry, ash-, and protein-free XSc marc. The results are presented in Tables XV and XVI and Figure 6.

The "Growth Increment" values in Table XV were obtained by dividing each "Carbohydrate Plus Lignin" figure by the 100 grams of XSc marc (the basis upon which the original calculation were made). The values in Table XVI were obtained by dividing the corresponding values in the preceding table by the total amount of that particular component deposited by the 53 growth stage. For example, the SX lignin value in Table XVI was obtained by dividing 33.8 grams by 469 grams to give 7.2%.

These calculations show strikingly how very near to the cambium the XSc tissue must have been. Only about 5% of the total cell-wall material (carbohydrate plus lignin) had been deposited at this stage in growth. At the SX growth stage the cells were about 20% mature,

TABLE XV

WEIGHT OF COMPONENTS DEPOSITED AT EACH GROWTH STAGE[1]

| Growth Stage | Lignin, g. | Glucan, g. | Xylan, g. | Mannan, g. | Galactan,[2] g. | Araban,[2] g. | Methylated Uronic Anhydride, g. | Total Carbohydrate, g. | Carbohydrate Plus Lignin, g. | Growth Increment |
|---|---|---|---|---|---|---|---|---|---|---|
| XSc | 0.0 | 23.9 | 9.7 | 1.4 | 16.2 | 10.8 | 38.0 | 100.0 | 100.0 | 1.0 |
| SX | 33.8 | 231.0 | 95.9 | 5.9 | 16.2 | 10.8 | 59.4 | 419.0 | 454.0 | 4.5 |
| 54 | 324.0 | 942.0 | 340.0 | 28.8 | 16.2 | 10.8 | 124.0 | 1462.0 | 1786.0 | 18.0 |
| 53 | 469.0 | 1137.0 | 361.0 | 45.4 | 16.2 | 10.8 | 123.0 | 1693.0 | 2162.0 | 21.6 |

[1] All calculations were based on the ovendry, ash-, and protein-free marcs.

[2] The assumption is made that the galactan and araban were deposited entirely by the XSc growth stage.

TABLE XVI

PERCENTAGE OF COMPONENTS DEPOSITED AT EACH GROWTH STAGE

| Growth Stage | Lignin, % | Glucan, % | Xylan, % | Mannan, % | Galactan,[1] % | Araban,[1] % | Methylated Uronic Anhydride, % | Total Carbohydrate, % | Carbohydrate Plus Lignin, % |
|---|---|---|---|---|---|---|---|---|---|
| XSc | 0.0 | 2.1 | 2.7 | 3.1 | 100.00 | 100.0 | 30.9 | 5.9 | 4.6 |
| SX | 7.2 | 20.3 | 26.6 | 13.0 | 100.0 | 100.0 | 48.3 | 24.7 | 21.0 |
| 54 | 69.1 | 82.9 | 94.1 | 63.5 | 100.0 | 100.0 | 100.8 | 86.3 | 82.6 |
| 53 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

[1]The assumption is made that the galactan and araban were deposited entirely by the XSc growth stage.

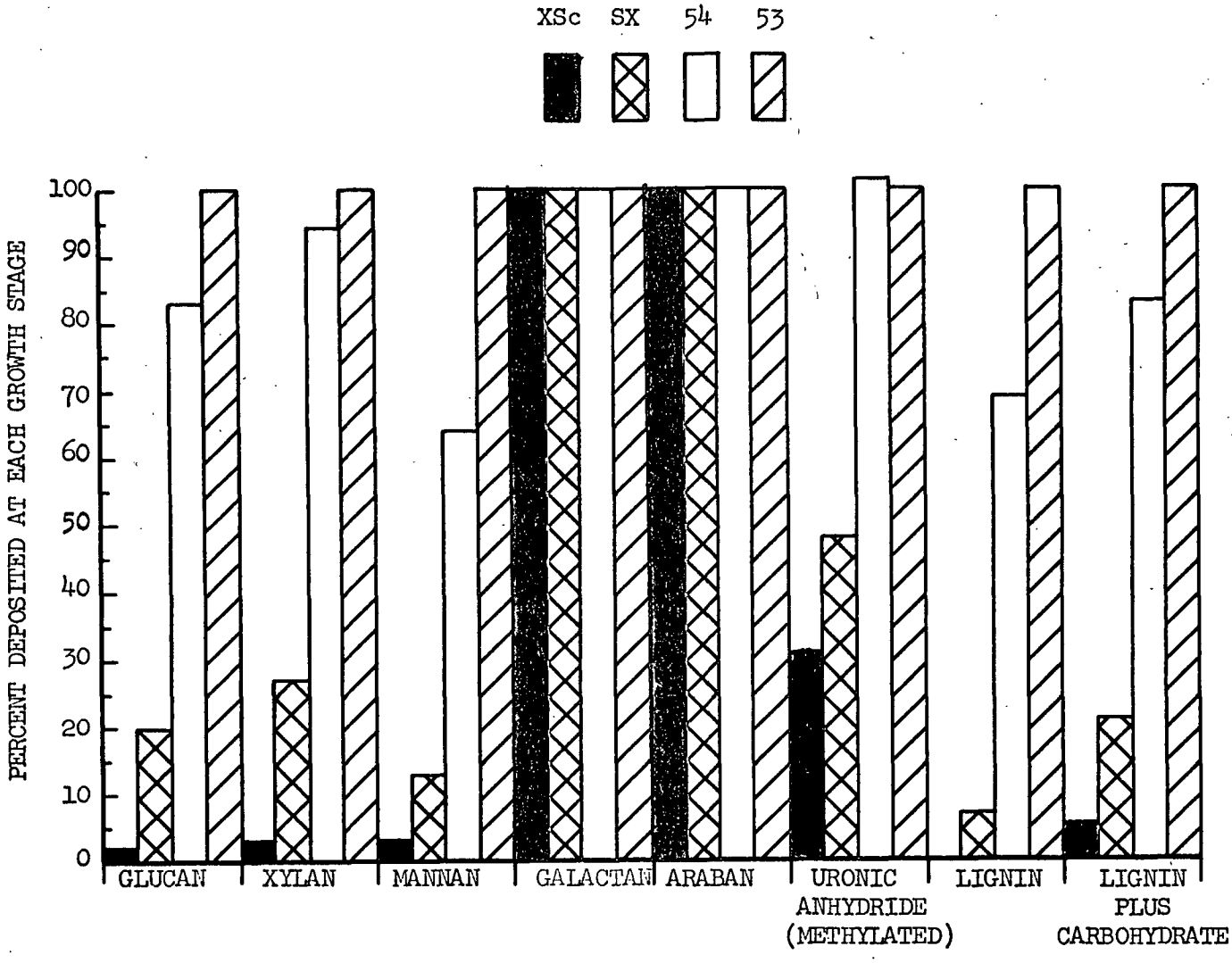


Figure 6

Percentage of Components Deposited at Each Growth Stage

and calculations based on the average composition of the 54 tissue show that it had grown to about 85% maturity. The 53 tissue, as mentioned above, may be considered completely mature.

Table XV shows that the total cell-wall material has increased 21.6 times from the XSc growth stage to maturity.

## APPROXIMATE DISTRIBUTION OF CARBOHYDRATES ACROSS AN AVERAGE CELL WALL

The morphological concept of cell-wall formation is that the polysaccharides are laid down centripetally as each cell matures. Thus, if the growth stages of Table XVI are pictured as steps in the maturation of a single, average aspen cell, the total amount of carbohydrate material deposited during each period can be represented as shown in Figure 7.

With this picture in mind, the data of Table XV can be used to determine the approximate composition of the successive cell-wall layers and hence the approximate distribution of carbohydrate constituents across an average cell wall. This has been done and is presented in Tables XVII and XVIII.

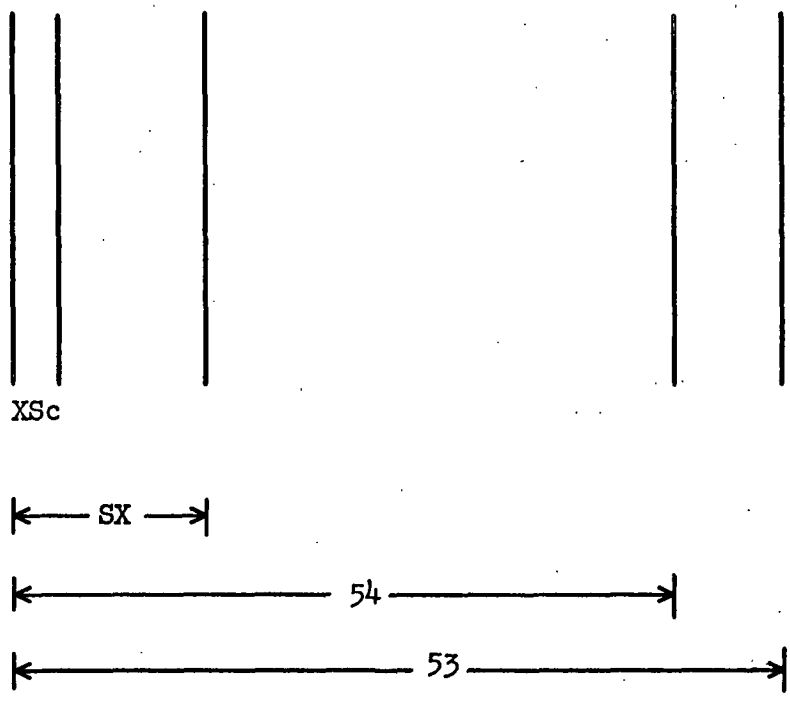


Figure 7

Schematic Representation of Average Aspen Cell Wall Showing Relative Amount of Carbohydrate Material Deposited During Each Growth Stage

TABLE XVII

CARBOHYDRATE WEIGHT COMPOSITION OF SUCCESSIVE CELL-WALL INCREMENTS[1]

| Growth Increment | Glucan, g. | Xylan, g. | Mannan, g. | Galactan,[2] g. | Araban,[2] g. | Methylated Uronic Anhydride, g. | Total Carbohydrate, g. |
|---|---|---|---|---|---|---|---|
| XSc | 23.9 | 9.7 | 1.4 | 16.2 | 10.8 | 38.0 | 100.0 |
| XSc--SX | 207.1 | 86.2 | 4.5 | 0.0 | 0.0 | 21.4 | 319.0 |
| SX--54 | 711.0 | 244.1 | 22.9 | 0.0 | 0.0 | 64.6 | 1043.0 |
| 54--53 | 195.0 | 21.0 | 16.6 | 0.0 | 0.0 | -1.0 | 232.0 |

[1]All calculations were made on the basis of the ovendry, ash-, and protein-free marcs.

[2]The assumption is made that the galactan and araban were deposited entirely by the XSc growth stage.

TABLE XVIII

PERCENTAGE COMPOSITION OF SUCCESSIVE CELL-WALL INCREMENTS

| Growth Increment | Glucan, % | Xylan, % | Mannan, % | Galactan,[1] % | Araban,[1] % | Methylated Uronic Anhydride, % | "Hemicellulose",[2] % |
|---|---|---|---|---|---|---|---|
| XSc | 23.9 | 9.7 | 1.4 | 16.2 | 10.8 | 38.0 | 76.1 |
| XSc--SX | 64.9 | 27.0 | 1.4 | 0.0 | 0.0 | 6.7 | 35.1 |
| SX--54 | 68.1 | 23.4 | 2.2 | 0.0 | 0.0 | 6.2 | 31.9 |
| 54--53 | 84.0 | 9.1 | 7.2 | 0.0 | 0.0 | -0.4 | 16.0 |

[1]The assumption is made that the galactan and araban were deposited entirely by the XSc growth stage.

[2]In this instance only, "hemicellulose" refers to those cell-wall polysaccharides which do not yield glucose on hydrolysis.

The values in Table XVII were obtained from Table XV by subtraction. For example, the XSc--SX glucan value in Table XVII was obtained by subtracting 23.9 grams (XSc, Table XV) from 231.0 (SX, Table XV) to give 207.1 grams. The values in Table XVIII were obtained from Table XVII by dividing the figure for a given constituent by the total carbohydrate laid down in the growth increment of interest. For example, the XSc--SX glucan value in Table XVIII was obtained by dividing 207.1 grams by 319 grams to give 64.9%.

The tables show that the glucan content of the cell wall sections increased as the inner layer of the cell wall was approached; the mannan content increased in the same manner reaching a maximum, like the glucan, in the cell-wall layer nearest the lumen. In fact the 54--53 increment was surprisingly rich in mannan. The xylan content increased to a mixiumum value in the middle two sections of the cell wall and then dropped off again in the section nearest the lumen. The uronic anhydride content declined quickly from a maximum value of 38% in the XSc increment to a value of 7 and 6% in the middle sections and finally to a negligible value in the layer nearest the lumen.

It can be seen from Table XVIII that the "hemicelluloses" were more concentrated in the outer layer of the cell wall, representing approximately 75% of the total carbohydrate material in this region and only about 15% of the total carbohydrate material in the layer nearest the lumen. These results are in very good agreement with

those obtained using microspectrographic techniques in which it was found that hemicellulose represented half or more of the carbohydrate material in the outer regions of the cell wall of spruce and birch and only 10-20% of the carbohydrate material around the lumen (47).

## ESTIMATED COMPOSITION OF INTERCELLULAR SUBSTANCE

In order to estimate the composition of the nonlignified true middle lamella or intercellular substance, the carbohydrate composition of the XSc and SX tissue was calculated on an ovendry, ash-, protein-, and lignin-free basis. In carrying out the calculations, the assumption was made that all the galactan, araban, and galacturonic anhydride had been deposited by the XSc stage and that the XSc uronic anhydride represented only galacturonic anhydride. The results of these calculations are shown in Table XIX.

TABLE XIX

CARBOHYDRATE COMPOSITION OF XSc AND SX MARCS[1]

| Tissue | Glucan, % | Xylan, % | Mannan, % | Galactan, % | Araban, % | Methylated Uronic Anhydride, % |
|---|---|---|---|---|---|---|
| XSc | 23.9 | 9.7 | 1.4 | 16.2 | 10.8 | 38.0 |
| SX | 55.2 | 22.9 | 1.4 | 3.9 | 2.6 | 9.1[2] |

[1]All calculations were made on the basis of the ovendry, ash-, protein-, and lignin-free marcs.

[2]This figure does not include the uronic anhydride deposited between the XSc and SX growth stages.

These data are plotted in Figure 8. The distance between XSc and SX is arbitrary and does not affect the results. Upon extrapolation, the glucan and xylan plots intersect at a point the locus of which is very nearly on the abscissa. If a vertical line through this point (the point at which glucan and xylan diminish to zero) represents the nonlignified intercellular substance, then the points at which the extrapolated galactan, araban, and galacturonic anhydride plots intersect it should indicate the composition of the intercellular substance. It is found, by this device, that the intercellular substance contains approximately 25% galactan, 17% araban, and 59% galacturonic anhydride.

To draw such conclusions from so little data is to be quite speculative. Still, the fact that the summation of the percentages thus obtained is very nearly 100% indicates that results are plausible. It is interesting that apple pectin has been found to contain 30% galactan, 20% araban, and 49% galacturonic anhydride (48).

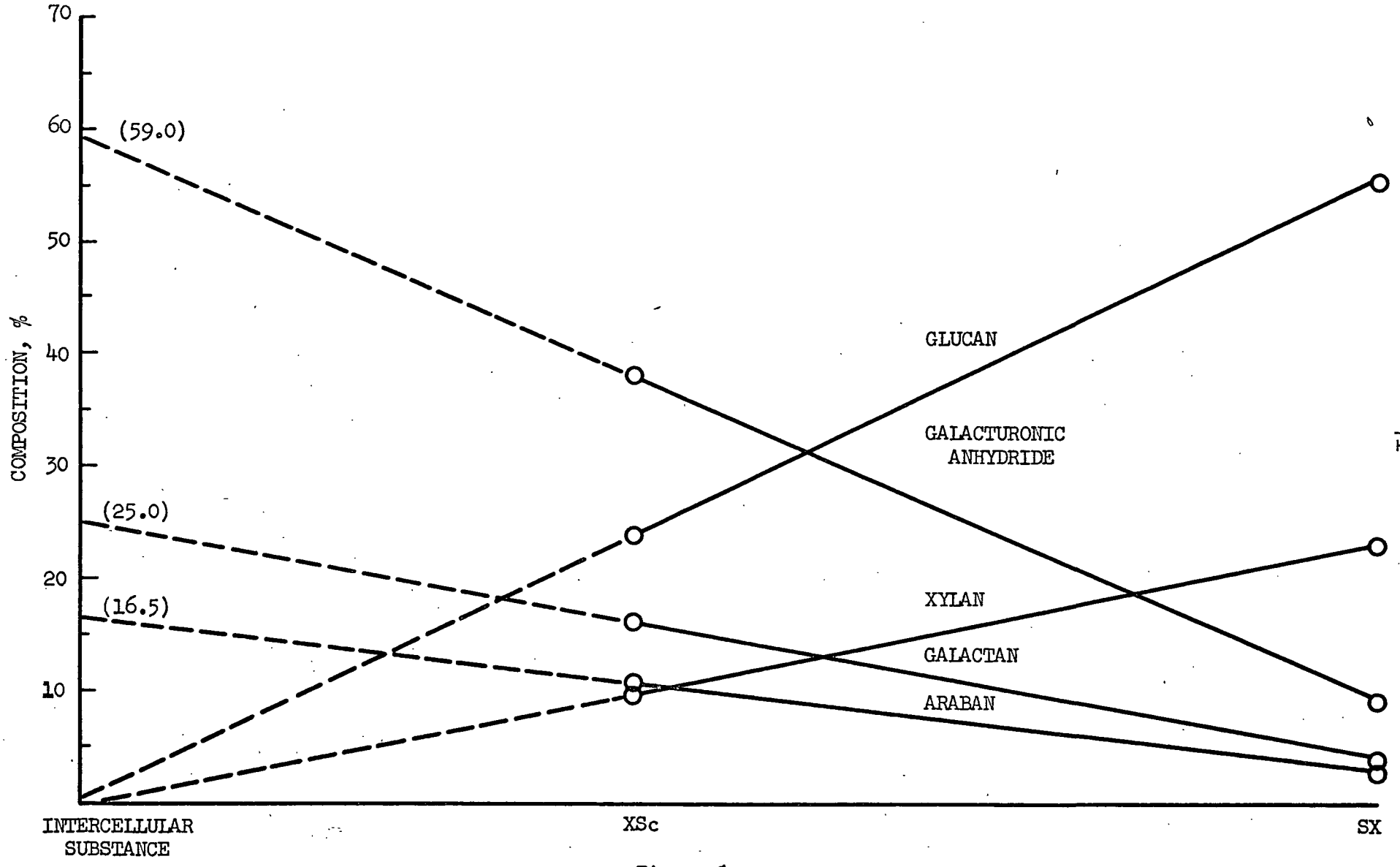


Figure 8

Graphic Estimation of Composition of Intercellular Substance

## EXTRACT STUDIES

The data in Table III, page 33, show that the SX tissues were very rich in 90% methanol-soluble extractives, an indication of high biochemical activity; in fact the extractives accounted for nearly half of the total solid material. The 54 and 53 tissues, on the other hand, were relatively poor in extractives for they contained only one-fourth and one-tenth as much 90% methanol-soluble material, respectively, as the SX tissues. The fact that 54 tissues contained significantly more extractives than the 53 tissues indicated that the former retained some biochemical activity throughout the growing season in which it originated.

It is interesting to note that 80-90% of the 90% methanol-soluble extractives in the SX and 54 tissues was water soluble. This is indicative of the abundance in these tissues of relatively simple compounds many of which probably are intermediates in the formation of the insoluble cell-wall components.

## PRELIMINARY EXAMINATION OF WATER- AND METHANOL-SOLUBLE FRACTIONS

The water- and methanol-soluble fractions (Figure 5, page 25) were examined by paper partition chromatography for specific carbohydrates and phenols. About 0.5 mg. of material from each fraction was spotted on Whatman No.1 chromatographic paper. The carbohydrate chromatograms were developed with BPW(6:4:3) for 24 hours, dried, and sprayed with p-anisidine hydrochloride. The phenol chromatograms

were developed with butanol-benzene-acetic acid-water (1:5:1:0.5) for eight hours, dried, and sprayed with ferric ferricyanide.

Generally speaking, the phenolic substances appeared in the methanol fractions while the carbohydrates appeared in the aqueous fractions, though the separation was not perfect.

Spots corresponding to glucose and sucrose* occurred in all aqueous fractions, sucrose being predominant. Fructose spots were also present in most of these fractions. The SX- and 54-methanol fractions contained relatively small amounts of material corresponding to glucose and sucrose and no spots corresponding to fructose.

No pentose sugars could be detected on the chromatograms. Pink spots, which could indicate pentoses, pentosans, or uronic acids, were evident at the top of the chromatogram in the SX-aqueous fractions. These were later shown to be acidic in nature.

The butanol-benzene-acetic acid-water did not resolve the phenolic materials. Streaks rather than distinct spots were obtained, although for some fractions spots could be seen near the solvent front.

It is interesting that of the three aldohexoses, two aldopentoses and rhamnose which represent fundamental structural units in the cell-wall carbohydrates of aspen only glucose could be detected in the extracts

*The presence of sucrose in the extract of the developing tissues of Populus grandidentata has been established by preparation of the octa-acetate derivative (49).

by paper chromatography. The presence of sucrose and some fructose was also indicated, sucrose always being the predominant sugar. This suggests that these sugars are the soluble carbohydrate reserve of the tree. Apparently they are in enzymatic equilibrium with starch, the insoluble carbohydrate reserve. The insoluble form is necessary because osmotic pressure considerations limit the amount of sucrose, glucose, and fructose that can be present in the cell sap.

Evidently in aspen the sucrose-starch system is the primary system from which all cell-wall carbohydrates are formed. It is known that four of the hexoses (glucose, fructose, mannose, and galactose) can be transformed into sucrose in plants (50). Hence, it is not unreasonable to assume that the reverse is true, i.e., that mannose and galactose as well as glucose and fructose can be formed from sucrose. Moreover, it is possible that glucuronic and galacturonic acids are formed from the corresponding hexoses by oxidation and that decarboxylation of the acids produces xylose and arabinose, respectively. However, the transformations in the conversion of substances such as these into the polysaccharides of the cell wall have not yet been elucidated.

It is also interesting that no low DP carbohydrates which might be considered intermediates in the formation of the high DP cell-wall carbohydrates could be detected in the extracts. Apparently the kinetics of the reactions by which cell-wall carbohydrates are formed are such that the intermediate substances cannot be detected by the usual methods, or perhaps they are present in the form of

complexes which do not lend themselves to chromatographic techniques such as those used in this study. This suggests that special experiments should be designed with the objective of maximizing the yield of the suspected intermediate compounds.

## STUDY OF WATER-SOLUBLE FRACTION

Since the preliminary chromatographic investigations had shown that the composition of the four SX-aqueous solutions and the composition of the four 54-aqueous solutions was qualitatively the same, they were combined to give one SX-aqueous solution and one 54-aqueous solution. A few introductory experiments indicated that an examination of the water-soluble acidic materials might be fruitful. In order to simplify the study of these materials by chromatographic techniques, it was necessary to separate them from glucose, sucrose, and fructose which were the predominant water-soluble constituents. This was accomplished by adsorbing the acids on a column of IR-4B resin. The acetate form of the resin was used instead of the hydroxide form because of the tendency of the latter to degrade reducing sugars to acids (51).

The column was washed with water until a small sample of the eluate gave a negative Molisch test, indicating that all the sugars had been removed from the resin. The acids were then eluted with 1N sulfuric acid, the acid eluate was neutralized with barium carbonate, and the barium sulfate was filtered off. After passing the acids through an IR-120 column to remove barium, the eluate was concentrated in vacuo.

The SX-sugar eluate gave a negative Maüle test and a negative Wiesner test. The 54-sugar eluate gave a positive Maüle test and a negative Wiesner test. These solutions were then covered with toluene and stored in the deep freeze, but were not investigated further.

The SX-acid solution gave a positive Maüle test and a cream-colored precipitate with the Wiesner reagent. The Maüle-positive material was not investigated further. The acids were chromatographed for forty-eight hours in EAW (9:2:2). Upon spraying with p-anisidine hydrochloride a definite pink spot was noted near the bottom of the chromatogram indicating the presence of a uronic acid. In ethyl acetate-acetic acid formic acid-water (18:3:1:4), the compound had a $R_f$ of about 0.50.

Since phosphate sugar esters are thought to be intermediates in the formation of some polysaccharides, the SX acids were examined chromatographically for phosphate compounds using phosphoric acid as a control. Two phosphate compounds were detected by spraying the chromatogram with a molybdic acid solution (52). These compounds had $R_f$ values that were less than that of phosphoric acid.

The identity of the indicated uronic acid compound and the two phosphate compounds remains to be established.

The 54 acids gave a positive Maüle test and a positive (red-violet) Wiesner test. It was found that the Maüle- and Wiesner-positive materials could be separated chromatographically with the butanol-acetic acid-water (4:1:5) solvent system. Three Maüle-

positive materials were detected, one of which remained at the starting line. One fluorescent substance with an $R_f$ value of about 0.85 gave both a positve Mäule and a positive (red-violet) Wiesner test. More than 20 different solvent systems were tried in an unsuccessful attempt to resolve this Mäule-positive, Wiesner-positive material into two or more compounds. It acted very much like a single substance under these conditions.

Chromatographic evidence indicated that this substance did not contain carboxylic acid groups. The 54 acids were developed in ethanol-water-concentrated ammonium hydroxide (70:30:5.6) and the chromatogram was sprayed with an indicator consisting of 50 mg. of bromphenol blue and 200 mg. of citric acid in 100 ml. of water (53). Because of the buffer capacity of the acid anions, the location of the spots was shown by an intense blue (alkaline) color against an orange-yellow background. The Mäule-positive, Wiesner-positive substance did not give the blue color.

As far as can be determined there has been no previous report in the literature of the separation from developing woody tissue of a substance giving these two color reactions which are typical of hardwood lignin. If the material is indeed a precursor of hardwood lignin, the fact that it is water soluble would indicate a relatively low molecular weight as compared to native hardwood lignin.

## STUDY OF METHANOL-SOLUBLE FRACTION

Only a very cursory examination of the methanol-soluble ex-

tractives was made. Since the preliminary chromatographic investigation had shown that the composition of the four SX-and 54-methanol solutions was qualitatively the same, they were combined to give one methanol solution. This was concentrated in a 40°C. water bath *in vacuo* to a thick sirup, and finally to dryness *in vacuo* at room temperature.

The residue was treated with ether and centrifuged to clarify. The ether solution was concentrated at room temperature. After most of the ether had been removed, a thick sirup remained. The ether-soluble material gave a negative Maüle test and pink Wiesner test. The material was not investigated further.

The ether-insoluble material was dried and a small portion was taken up in methanol and chromatographed in BPW (6:4:3). Most of the phenolic material appeared at a spot very near the solvent front. The spot was somewhat diffuse, gave a blue-green fluorescence under ultraviolet light, gave characteristic Maüle and Wiesner tests, and gave a dark purple color with diazotized *p*-nitroaniline. Aspen native lignin had the same $R_f$ in the BPW (6:4:3), but gave a yellow-orange fluorescence under ultraviolet light.

## OTHER EXPERIMENTS

### PAPER FROM MACERATED SOFT XYLEM

It is necessary to delignify mature wood tissue by relatively drastic chemical treatment in order to separate it into individual fibers from which paper can be made. This treatment necessarily results in a certain amount of attack on the fibers themselves causing a loss in the strength properties of the paper made from them.

It was speculated that paper made from wood tissue which had not been lignified and therefore did not have to be subjected to the usual pulping and bleaching operations to remove lignin might indicate the maximum strength properties obtainable from the particular species being investigated. Since the aspen SX tissue had a very low lignin content, attempts were made to macerate it by a very minimum of mechanical or chemical treatment.

In the first experiment, mechanical maceration alone was investigated. The SX shavings were cut into short strips and soaked at 10% consistency for about 60 hours. The tissues were then beaten in a Jokro mill at 6% consistency. Handsheets were formed and tested (54). The results are recorded in Table XX.

TABLE XX

HANDSHEET PROPERTIES OF MECHANICALLY MACERATED SX TISSUE

| | Run A | Run B |
|---|---|---|
| Beating time, min. | 30 | 45 |
| S.-R. freeness, cc. | 585 | 550 |
| 25 x 40--500 basis weight, lb. | 48.9 | 49.2 |
| Apparent density | 15.3 | 14.9 |
| Burst factor, points/100 lb. | 102 | 94.1 |
| Tear factor | 0.36 | 0.37 |
| Tensile strength, lb./inch | -- | 17.0 |
| Folding endurance | 380 | -- |

The strength properties of these handsheets are somewhat lower than those expected from a bleached neutral sulfite semichemical aspen pulp. When viewed under a microscope, the beaten fibers showed little evidence of fibrillation. The fibers were short and appeared to be pulled apart and cut rather than brushed. The handsheets had much "rattle", were brittle, and were translucent. The formation in the handsheets was poor and there appeared to be many small clumps of fibers, indicating poor maceration by mechanical treatment alone. The handsheets had a very low porosity of about 50 cc. of air in 24 hours as measured with a Gurley Densometer.

No further handsheet studies were made. However, several mild chemical treatments of the tissue were made to study chemical maceration. The most successful involved treating the SX shavings for two one-hour periods with acidified sodium chlorite (55) to remove lignin, followed by two one-hour treatments with 0.5% ammonium oxalate at 85°C. to remove pectic materials. Microscopic examination showed that most of the shavings had been defiberized and that there was no visible damage to the fibers.

## INDICATOR USED IN QUANTITATIVE SUGAR DETERMINATIONS

Methyl red (29) and screened methyl red (30) are the indicators most commonly used in the titration of formic acid produced by the oxidation of simple sugars with sodium metaperiodate in quantitative sugar determinations. The former was used in this study with good accuracy (see Table XI, page 49). However, the end point was extremely difficult to see even when color comparisons were made with a blank. Consequently, much practise was required to attain consistently accurate results and the titrations were time consuming.

A search for a better indicator revealed that bromcresol green had a sharper end point at the desired pH than either methyl red or screened methyl red. A blank containing the indicator, distilled water, and sodium metaperiodate was made up to a volume approximately equal to that of the unknown solutions, and was titrated to a pH meter reading of 5.5 with a very weak solution of sodium hydroxide. The blank could be kept at room temperature for at least 24 hours without a change in pH or color. The unknown solutions were then titrated to the end point indicated by the blank. Such titrations were rapid and caused much less eyestrain.

In order to compare the results obtained using the methyl red indicator with those obtained using the bromcresol green indicator, quantitative sugar determinations were made on a hydrolyzed wood sample. The result are given in Table XXI.

TABLE XXI

CARBOHYDRATE COMPOSITION OF WOOD SAMPLE AS DETERMINED USING BROMCRESOL GREEN AND METHYL RED INDICATORS

| | Bromcresol Green | Methyl Red |
|---|---|---|
| Glucan, % | 41.3 | 41.1 |
| Xylan, % | 15.3 | 16.2 |
| Mannan, % | 1.2 | 1.4 |
| Galactan, % | 0.5 | 0.7 |
| Araban, % | 0.2 | 0.4 |

It can be seen that both indicators gave nearly the same results. The bromcresol green indicator also produced more precise results.

## GENERAL SUMMARY

Newly formed tissues were collected from aspen trees cut at four dates spanning most of the 1954 growing season. Tissues at successive growth stages were removed from the barked logs at each date. The youngest tissue, the soft xylem, was the thin layer of xylem removed from the face of the log; the 1954 sapwood was the more fully differentiated xylem located between the soft xylem and the 1953 growth ring; the 1953 sapwood was the completely differentiated xylem located between the 1954 sapwood and the 1952 growth ring. In June of the following year, a small amount of extremely young tissue, termed xylem scrapings, was collected.

The developing tissues were removed from the logs and were immersed in absolute methanol immediately after the trees had been felled. After the first two field trips, the billets from which the soft xylem and 1954 sapwood had been removed were brought to the laboratory where the 1953 sapwood was removed, air dried, and comminuted to pass a 10-mesh screen before being immersed in methanol.

Karl Fischer moisture determinations carried out on separate samples indicated that the soft xylem and 1954 sapwood tissues were very succulent and that the moisture content of these materials decreased as the season progressed.

The soft xylem, 1954 sapwood, and 1953 sapwood tissues were extracted for four successive four-day periods with 90% methanol,

the extracts being reserved for further study. The xylem scrapings extracts, obtained by extracting the tissue twice for one-hour periods with 90% methanol under reflux, were not investigated.

All marcs were air dried, comminuted to pass a 40-mesh screen, and analyzed for moisture, ash, nitrogen, uronic anhydride, methoxyl, lignin, and cell-wall carbohydrates. There was some indication of a variation in the composition of the soft xylem and 1954 sapwood marcs as the growing season progressed. However, this was not attributed to a difference in composition between spring- and summerwood tissues. The 1953 sapwood had nearly the same composition as the whole wood and was, therefore, considered mature.

Assays of the 1954 sapwood showed it to be transitional in growth between the soft xylem and the 1953 sapwood. The soft xylem and xylem scrapings were characterized by a lower glucan, mannan, lignin, and methoxyl content, and a higher uronic anhydride, galactan, araban, and protein (nitrogen x 6.25) content than the 1954 and 1953 sapwood.

The xylem scrapings were remarkable in that they apparently were nonlignified, exhibited little or no secondary thickening, and were very high in pectic substances (galactan, araban, and uronic anhydride) and very low in glucan (15%). The composition of the xylem scrapings seems to approach that of the cambium.

By making the assumption that the pectic substances were deposited completely in the material represented by the xylem scrapings,

it was possible to estimate the growth imcrements of the carbohydrate constituents and lignin as the tissue matured. It was also possible to approximate the distribution of the carbohydrate constituents across an average aspen cell wall and to estimate the compositon of the nonlignified true middle lamella.

The soft xylem, 1954 sapwood, and 1953 sapwood methanol extracts were each separated into two main fractions. An aqueous solution was obtained by concentrating the respective extracts in vacuo to an aqueous suspension which was then filtered through a celite-asbestos pad. A methanol solution was obtained by thoroughly extracting the pad with absolute methanol.

The methanol solutions were given a cursory chromatographic examination which indicated the presence of materials giving lignin color reactions. Chromatographic studies of the aqueous solutions showed that sucrose, glucose, and fructose were abundant in the extracts of the developing tissues, sucrose always predominating. Among the water-soluble acids which were separated by ion-exchange techniques, the presence of two organic phosphate compounds, a uronic acid, and several materials giving lignin color reactions was indicated chromatographically. Of particular interest was one substance, extracted from the 1954 sapwood, which gave characteristic Maüle and Wiesner tests and appeared to be a single compound.

## CONCLUSIONS

The conclusions derived from this investigation may be stated as follows:

1. Aspen tissues at successive stages of maturity can be obtained in a form suitable for analysis throughout a normal growing season.
2. The tissue formed in a given growing season can be obtained in at least three successive stages of maturity, viz., a colloidal mass of tissue scraped lightly from the log with a knife blade held perpendicular to the surface (xylem scrapings); a thin, translucent tissue resembling onion skin obtained by drawing a knife blade lengthwise along the face of the log in such a way that it is nearly parallel to the log surface (soft xylem); and more less thick shavings containing more fully differentiated elements removed from the log with a drawshave (1954 sapwood).
3. A very thin layer of pale-yellow tissue composed of summerwood elements is located at the boundary between the annual increments of aspen sapwood.
4. The developing tissues of aspenwood are extremely succulent, water being the major constituent during the growing season.
5. The 90% methanol-soluble extractives can be removed nearly quantitatively by four successive four-day extractions at room temperature.
6. Although there was some indication of a variation in the composition of the soft xylem tissues from early June to

mid-August, the methods of collection were not sufficiently precise to conclude that it was due to a difference in composition between spring- and summerwood.

7. The 1953 growth ring had very nearly the same composition as the whole wood.

8. Assays of the 1954 sapwood indicated that it was much more mature than the soft xylem, but that it had not yet reached the full maturity of the 1953 sapwood.

9. The youngest aspen tissues (xylem scrapings and soft xylem) differ in chemical composition from mature tissue in that they have:

   (a) A higher moisture content,

   (b) A higher extractives content,

   (c) A higher nitrogen content,

   (d) A higher uronic anhydride content,

   (e) A higher galactan content,

   (f) A higher araban content,

   (g) A lower glucan content,

   (h) A lower xylan content (xylem scrapings only),

   (i) A lower mannan content,

   (j) A lower methoxyl content, and

   (k) A lower lignin content

10. Chromatographic evidence indicates that all monosaccharides considered constituents of mature aspen cell-wall polysaccharides as well as traces of fucose and ribose (the latter probably arising from nucleic acids in the protoplasm) are present in the xylem scrapings hydrolyzate.

11. The xylem scrapings are extraordinary in that they:

    (a) Give no qualitative or quantitative evidence of being lignified,

    (b) Exhibit little or no secondary thickening,

    (c) Have a very high percentage of pectic substances (galactan, araban, and uronic anhydride), and

    (d) Have a very low percentage of glucan.

12. The composition of the xylem scrapings, therefore, seems to approach that of true cambial tissue.

13. The data suggest the hypothesis that the pectic substances are deposited completely by the xylem scrapings growth stage.

14. By using araban (one of the pectic substances) as an internal index of cell-wall growth, it is possible to estimate the quantity of carbohydrate constituents and lignin deposited between the successive growth stages relative to 100 grams of ovendry, ash-, and protein-free xylem scrapings.

15. The following conclusions are based on the internal index concept:

    (a) The absolute quantity of cell-wall material (carbohydrate plus lignin) increases about 20 fold from the xylem scrapings growth stage to the 1953 growth stage, i.e., from the cambium to maturity.

    (b) Only about 5% of the total cell-wall material was deposited by the xylem scrapings stage, 20% by the soft xylem stage, and 85% by the 1954 stage.

    (c) The percentage of glucan and mannan increases from a low value in the primary wall to a maximum in the cell-wall layer nearest the lumen.

    (d) The xylan percentage is highest in the middle of the cell wall, dropping off considerably in the primary wall and in the layer nearest the lumen.

    (e) The uronic anhydride content decreases rapidly

from a maximum in the primary wall to a negligible value in the layer nearest the lumen.

(f) A graphic estimation shows that the intercellular substance contains approximately 59% galacturonic anhydride, 25% galactan, and 17% araban.

16. Chromatographic evidence indicates that sucrose is the predominant constituent in the extractives of developing aspen tissues and that glucose and fructose are also present in considerable quantities.

17. Two organic phosphate compounds, a substance which gave a typical uronic acid color when sprayed on a chromatogram with p-anisidine hydrochloride, and a substance which gave characteristic Mäule and Wiesner tests were detected chromatographically among the water- soluble acids extracted from the developing tissues.

## SUGGESTIONS FOR FUTURE WORK

1. Although many of the results of a study of this nature apply to woody tissues in general, it is obvious that some are specific for the species investigated. Therefore, it would be desirable to extend cambial studies to a greater variety of hard- and softwood species. Indeed, it may be easier to study the formation of certain cell-wall constituents in softwoods than in hardwoods. For example, it may be easier to study the formation of softwood lignin because it contains guaiacyl groups whereas hardwood lignin contains both guaiacyl and syringyl groups and because the red-violet color of the Wiesner test (hardwoods and softwoods) appears to be more specific for lignin (or possible lignin precursors) than the pink to purple color of the Mäule test (hardwoods only).
2. Proteins play a vital role in plant metabolism. However, little is known concerning the part they play in cell wall formation other than the fact that enzymes act as catalysts for biochemical reactions. For example, it is possible that some intermediates are present in the form of complexes with proteins. It is also possible that certain amino acids are themselves intermediates in the formation of cell-wall constituents. An investigation of the cambial proteins could contribute much to our knowledge of the conversions leading to the formation of cell-wall polysaccharides and lignin.
3. No pentoses, hexoses (other than glucose and fructose), or low DP carbohydrates, which might be considered intermediates

in the formation of cell-wall polysaccharides, could be detected in the extracts of the developing tissues by the techniques used in this study. Hence, it would be desirable to make a detailed study of ways to maximize the yields of possible intermediate substances.

It is possible that the methods of collection used in this study were unfavorable from the standpoint of maximum yields of intermediate products. In order to minimize the time interval between collection and immersion of the samples in alcohol and to eliminate disturbance to the developing tissues, immediate immersion of growing tree seedlings in hot alcohol might be tried. Collecting materials at night rather than during the day or growing cambial tissues in vitro with special nutrients and/or under appropriate environmental conditions might increase the yields of certain intermediates. Tissue culture techniques in combination with methods such as those used in the study of photosynthesis to detect intermediates (56) (using radioactive glucose or sucrose instead of radioactive carbon dioxide as the source of $C^{14}$) might be adapted to the field of cambial chemistry. Such studies, for example, might lead to an understanding of transformations involved in the conversion of glucose into the pectic substances on the one hand and into cellulose, xylan, mannan, and glucuronic acid units on the other.

4. Experiments should be designed to establish whether or not the pectic substances are deposited completely and irreversibly

prior to secondary wall formation. One approach to the first condition would be to separate the primary and secondary walls of mature tissue and note the absence or presence of galactose, arabinose, and galacturonic acid in the hydrolyzate of the secondary wall. An approach to the second condition would be to determine quantitively the amount of galactose, arabinose, and galacturonic acid in the hydrolyzate of the secondary wall. An approach to the second condition would be to determine quantitatively the amount of galactose, arabinose, and galacturonic acid in the tissue before and after secondary wall formation. In a separate experiment, the weight in the tissue brought about by deposition of the secondary wall would have to be measured.

5. This study has indicated that there are relatively simple substances, presumably related to lignin, among the extractives of aspen tissue. It certainly would be desirable to isolate and characterize these substances and to consider them for inferences relating to the biogenesis and structure of aspen lignin. Moreover, the possibility of isolating and characterizing similar substances from the developing tissues of softwoods and other hardwoods should not be overlooked.

The writer cannot overemphasize his firm conviction that a great many questions concerning the conversions leading to the formation of lignin and cell-wall polysaccharides will be answered by future investigations in the field of cambial chemistry.

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in the very young sapwood, and from 3.85% in the inner bark to 7% in the cambial zone. Large amounts of crystalline sucrose were found in the cambial zone (33% on the dry basis) and in the inner bark. Pentosans, lignin, and methoxyl were low in the cambial zone but were present in large amounts in the outer bark, sapwood, and heartwood. Pectic materials occurred in large amounts in the inner bark where they were accompanied by starch.

5. Anderson, E., Wise, L. E., and Ratliff, E. K. Note on the chemistry of the cambial zone of black spruce. Tappi 37, no. 10:422-4(Oct., 1954); cf. preceding reference.

The cambial zone of black spruce contained 67% extractives on the dry basis, of which one-half was sucrose. Protein (7.0%) and pectin (3.0%) were also present in considerable quantities, whereas there was very little lignin (1.8%) and relatively small amounts of alpha-cellulose (11.9%) and other polysaccharides (4.9%).

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The authors used broken pieces of porcelain to remove cambial tissues from logs. Significant quantities of reducing sugars, oxalic, succinic, and citric acids, guaiacol, and vanillin were found in the saps of Picea abies, Pinus silvestris, and Fagus silvatica. The pentose content of all the saps was remarkably small, but greatest in beech.

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Moisture is extracted from the sample with absolute methanol. A known volume of Karl Fischer reagent (a methanol solution of iodine, sulfur dioxide, and pyridine) is then added to an aliquot of the extract and the mixture is titrated electrometrically with a methanol solution containing a predetermined amount of water until the "magic eye" control closes.

The method was modified as follows: A known volume of water-in-methanol solution was added to an aliquot of the extract and the mixture was titrated with the Karl Fischer reagent until the "eye" was open.

The amount of water in the samples was calculated from the relation,

$$\text{g. of water} = (KF \times R - MW)\ (F_1 \times W) - (b \times F_2 \times W),$$

where

KF = ml. Karl Fischer reagent to titrate the sample;
R = MW/KF;
MW = ml. water-in-methanol solution added;
$F_1$ = volume of methanol used as solvent/volume of extract aliquot;
w = water factor of MW in g. water per ml. MW;
b = (KF x R - MW) for blank; and
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The test sample is dried in an oven at 103-105°C. until it reaches constant weight.

17. Institute Method 4. Ash in wood. Appleton, Wis., The Institute of Paper Chemistry, 1951.

The sample is ignited carefully, first at a low tem-

perature to remove all volatile material and finally at 550°C. until all the carbonaceous material is removed. It is then cooled in a desiccator and weighed.

18. Institute Method 705b. Determination of Kjeldahl nitrogen (Hengar technique). Appleton, Wis., The Institute of Paper Chemistry, 1947.

The sample (100 mg.) is digested with concentrated sulfuric acid and potassium sulfate in the presence of a selenium catalyst. After the reaction mixture is cooled and made alkaline with sodium hydroxide, the ammonia is distilled off and collected in dilute sulfuric acid. The excess acid is titrated with sodium hydroxide.

19. Institute Method 25. Uronic acids. Appleton, Wis., The Institute of Paper Chemistry, 1951.

The sample is digested with 12% hydrochloric acid saturated with sodium chloride. The uronic acid is converted to furfural and carbon dioxide. The latter is swept out of the reaction vessel with a stream of nitrogen and collected quantitatively in an Ascarite-Anhydrone absorption tube. The uronic anhydride is calculated as $CO_2 \times 4$.

20. Institute Method 18. Methoxyl groups in wood. Appleton, Wis., The Institute of Paper Chemistry, 1951.

The material is digested with hydriodic acid, phenol, and hypophosphorous acid. The alkyl iodide thus formed is swept by a stream of carbon dioxide into a receiver containing an acetic acid solution of potassium acetate to which bromine has been added. After the iodide has been converted to iodic acid, the excess bromine is destroyed with formic acid. The iodine liberated upon the

addition of acidified potassium iodide is then titrated with sodium thiosulfate.

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The authors studied the formation of lignin in bamboo by alkaline nitrobenzene oxidation. A considerable amount of vanillin and p-hydroxybenzaldehyde but only a small amount of syringaldehyde were found in bamboo shoots. In mature bamboo, however, the syringaldehyde predominated.

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From the data of Thomas (Table XII, page 57) a glucan value of 49.1% can be calculated as follows: The holocellulose yield was 77.8% of which 65.3% was alpha-cellulose. The latter contained 1.4% xylan and about 2% mannan. Thus,

77.8% x .653 = 50.8% glucan (uncorrected)

50.8%(.014 + .02) = 50.8% x .034 = 1.7% (mannan and xylan)

50.8% - 1.7% = 49.1% (corrected for mannan and xylan).

37. Wise, L. E., and Jahn, W. C. Wood chemistry. 2d. ed. Vol. 1, p. 1264. Data from an unpublished Forest Products Laboratory report. New York, Reinhold, 1952.

In analyses made on two aspen samples on the original wood basis, alpha-cellulose values of 48.8% and 50.1% were found.

38. Schoettler, J. R. A study of the alkali resistance of the pentosans in aspenwood. Doctor's Dissertation. Appleton, Wis., The Institute of Paper Chemistry. 1952. 121p.

It was found that aspenwood meal contained 50.8% xylan-free alpha-cellulose.

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water to remove pectins. From the experimental data it is concluded that most of the galacturonic acid is formed within the cell itself, and not in the cell wall.

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The inner and outer barks of the flax stalk were analyzed during various stages of growth for pectic acid, pentosans, uronic acids, hemicelluloses, and cellulose. The inner fibrous bark contained more uronic acids than the outer wooden bark. During growth the extractable uronic acid content decreased, especially in the outer bark, and the residual uronic acid increased.

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The author reviews the chemistry and physiology of the pectins.

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Pectic materials were isolated from the cambial layer and sapwood of black locust. The composition of some of these substances approximated that of certain pectinic acids. "It

appears that the pectic materials in wood are deposited in the middle lamella and the primary cell wall in the early stages of cell development and remain even in the old wood."

44. Anderson, E., Seigle, L. W., Krznarich, P. W., Richards, L., and Marteny, W. W. The isolation of pectic substances from wood. II. J. Biol. Chem. 121, no. 1:165-74(Oct., 1937).

Pectic materials were isolated from mature hardwoods and softwoods. They were present in larger amounts in hardwoods than in softwoods. "Pectic materials in mature wood are apparently laid down early in the growth of the tissue and remain unchanged as the wood ages. They do not seem to be converted to lignin or hemicelluloses in wood."

45. Bailey, I. W., and Kerr, T. The cambium and its derivative tissues. X. Structure, optical properties, and chemical composition of the so-called middle lamella. In Bailey's Contributions to plant anatomy. p. 57. Waltham, Mass., Chronica Botanica, 1954.

"...the isotropic intercellular substance of mature wood appears to be composed of two substances, lignin and polyuronides, which may be separated by their differential solubilities. Similarly, "...the cambial walls of the mature wood appear to be composed of a mixture of lignin, cellulose, and polyuronides."

46. Bryan, W. H., and Newcomb, E. H. Stimulation of pectin methylesterase activity of cultured tobacco pith by indoleacetic acid. Physiol. Plantarum 7:290-7(1954).

"It is therefore attractive to assume that pectin methylesterase by controlling the rate and extent of demethylation of protopectin in the wall, may regulate polygalacturonase activity, and that these two enzymes are among those whose activities are controlled by auxin and which collectively make possible the plastic

stretching of the cell wall and the insertion of new material."

47. Lange, P. W., and Asunmaa, S. The distribution of "cellulose" and "hemicellulose" in the cell wall of spruce, birch, and cotton. Svensk Papperstidn. 57, no. 14:501-16(July 31, 1954).

The authors used an optical density method to determine the distribution of components across the cell wall. They found that "cellulose" was most densely packed around the lumen in spruce and birch with the relative packing density in the outermost layers roughly about half that around the lumen. "Hemicellulose" was about half or more of the carbohydrate material in the outer regions of the cell wall. Around the lumen the percentage of carbohydrate material consisting of "hemicellulose" was estimated at 10-20%.

48. Hirst, E. L., and Jones, J. K. N. Pectic substances. III. Composition of apple pectin and the molecular structure of the araban component of apple pectin. J. Chem. Soc. 1939:454-60.

Apple pectin was found to contain 49.2% anhydrogalacturonic acid, 20% araban, and about 30% galactan. The main features of the structure of apple pectin araban were identical with those of the araban isolated from peanut pectin.

49. Kremers, R. E. Unpublished data, 1954.

Sucrose was extracted from the developing tissues of Populus grandidentata and identified by preparation of the octa-acetate derivative.

50. McCready, R. M., and Hassid, W. Z. Transformation of sugars in excised barley shoots. Plant Physiol. 16:599-610(1941).

It was shown that glucose, fructose, mannose, glactose, glyceraldehyde, maltose, and lactose could be transformed into

sucrose by barley plants. However, the plants could not utilize arabinose, xylose, mannitol, sorbitol, gluconic acid, or pyruvic acid for the synthesis of sucrose.

51. Phillips, J. D., and Pollard, A. Degradation of sugars on ion-exchange columns of Amberlite IRA 400($OH^-$). Nature 171, no. 4340:41-2(Jan. 3, 1953).

It was found that there was considerable degradation of glucose and fructose to organic acids by the hydroxyl form of the resin; but there was no degradation when the resin was used in the carbonate form.

52. Hanes, C. S., and Isherwood, F. A. Separation of the phosphoric esters on the filter paper chromatogram. Nature 164, no. 4183: 1107-12(Dec. 31, 1949).

The method used for detecting phosphoric acid esters was as follows: the air-dried chromatogram was sprayed with a solution consisting of 5 ml. of 60% W/W perchloric acid, 10 ml. of 1N hydrochloric acid, 25 ml. of 4% W/W ammonium molybdate, and 60 ml. of distilled water. The paper, after drying in a current of warm air for a few minutes to remove excess water, was heated for 7 minutes at 85°C. To develop the color fully the paper was allowed to regain moisture from the air and was then hung for five to ten minutes in a jar containing dilute hydrogen sulfide gas. The esters appeared as intense blue spots against a faint buff background.

53. Kennedy, E. P., and Barker, H. A. Paper chromatography of volatile acids. Anal. Chem. 23, no. 7:1033-4(July, 1951).

The volatile acids were separated chromatographically as the corresponding ammonium salts using a solvent system consisting of 100 ml. of 95% ethanol to which 1 ml. of concentrated

ammonium hydroxide was added. The acids were located by spraying with a solution of 50 mg. of bromphenol blue in 100 ml. of water, and made acid with 200 mg. of citric acid. The acids appeared as intense blue spots against an orange-yellow background.

54. Institute Method 411. Forming and testing handsheets for physical tests of pulp. Appleton, Wis., The Institute of Paper Chemistry, 1951.

The procedure for forming handsheets from pulp is given, and the methods for determining the physical properties of the handsheets are described.

55. Wise, L. E., Murphy, M., and D'Addieco, A. A. Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. Paper Trade J. 122, no. 2:35-43(Jan. 10, 1946).

The preparation of chlorite holocellulose is described and the method is critically discussed.

56. Calvin, M. The path of carbon in photosynthesis. Chem. Eng. News 31, no. 16:1622-5(April 20, 1953).

Green algae were placed in a flat circular vessel and exposed to filtered light. Labeled carbon dioxide was bubbled into the apparatus and at suitable intervals of time the algae suspension was drawn into alcohol. Thus, photosynthesis with labeled carbon dioxide could be stopped after extremely short time intervals with a minimum of disturbance to the system. The alcohol extracts were then analyzed to determine which compounds contained radiocarbon and the order of appearance of radioactivity in the compounds.

# APPENDIX I

## RESULTS OF QUALITATIVE TESTS RUN ON MATERIAL PRECIPITATED ON CONCENTRATION OF TISSUE EXTRACTS*

Table XXII shows the results of quantitative tests run on the precipitated fractions. All fractions were examined under the microscope. Fractions 54-3 and 54-4 were observed to be predominantly fibrous, so they were discarded. No insoluble material separated during the concentration of the 53-2 extract. In making the final concentration of the 53-3 extract a brown material insoluble in water, methanol, and hexane separated. This material gave positive Molisch, Mäule, and Wiesner tests whereas the precipitate which separated earlier gave negative responses to these three tests. The former was labeled 53-3B and the latter 53-3A.

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*This is the water-, methanol-, and hexane-insoluble material that precipitated upon concentration of the tissue extracts (pages 27, 28, 29).

TABLE XXII

RESULTS OF QUALITATIVE TESTS AND YIELD DETERMINATIONS MADE ON MATERIAL PRECIPITATED ON CONCENTRATION OF TISSUE EXTRACTS

| | SX | | | | 54 | | | | 53 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3A | 3B | 4 |
| Molisch test | + | + | + | - | - | + | -- | -- | + | -- | - | + | - |
| Maule test | - | - | - | - | Faint + | + | -- | -- | + | -- | - | + | + |
| Wiesner test | - | + | + | + | Faint + | + | -- | -- | + | -- | - | + | + |
| Yield, g. | 0.7 | 2.2 | 3.5 | 0.5 | 0.8 | 0.9 | -- | -- | 2.6 | -- | 0.1 | 0.4 | 0.2 |

# APPENDIX II

## DESCRIPTION OF QUALITATIVE TESTS USED

Because some qualitative tests may be carried out in any one of several different ways, the methods used in this study are described below.

1. Molisch Test for Carbohydrate Materials

Three drops of a 10% solution of alpha-naphthol in 95% ethanol were shaken with about 1 ml. of an aqueous solution or suspension of the material under investigation in a small test tube. Concentrated sulfuric acid was added dropwise on the side of the test tube until a layer of the acid was visible at the bottom of the tube. A red to violet ring which developed at the interface between the two liquids within five minutes was considered a positive test.

2. Mäule Test

Three drops of chlorine water, prepared by bubbling chlorine into water, were shaken with about 1 ml. of the solution being tested. The chlorination was allowed to proceed for about five minutes and several crystals of sodium sulfite were added. A pink to red color which developed almost immediately at the surface of the crystals was considered a positive test.

In the case of tissue or other solid material, a few drops of concentrated ammonium hydroxide were added following chlorination with chlorine water. A red to purple color was considered a positive test.

In chromatographic studies, the dry chromatogram was

moistened over steam and placed in a closed beaker containing chlorine gas. After a ten-minute chlorination, the chromatogram was removed from the beaker and sprayed with a 10% solution of sodium sulfite. The test was considered positive upon immediate appearance of a pink spot or spots which soon faded.

3. Wiesner Test

In the case of solutions, three drops of 12% hydrochloric acid were shaken with about 1 ml. of the solution. Three drops of a 1% solution of phloroglucinol in 12% hydrochloric acid were then added and the mixture was well shaken. A red-violet color which developed within two minutes after the addition of the phloroglucinol was considered a positive test. A pink or red-brown color was not considered a positive test; however, the color was recorded.

In the case of tissue or other solid material, the reagents were added (in the same sequence) directly to the sample being tested. A red-violet color was considered a positive test.

In chromatographic studies, the dry chromatogram was sprayed with 12% hydrochloric acid, dried, and sprayed with the 1% phloroglucinol solution. The test was considered positive upon the appearance of a red-violet spot or spots.

4. Ferric Chloride Test for Phenols

Three to five drops of a 1% aqueous solution of ferric chloride were added to about 1 ml. of an aqueous or alcoholic solution of the material. The presence of a phenolic compound

or compounds was indicated by a change in color of the solution.

5. Naphthoresorcinol Test for Uronic Acids

One-half cubic centimeter of 1% naphthoresorcinol in alcohol and 0.5 ml. of concentrated hydrochloric acid were added to 5 ml. of the material in a test tube. The solution was then heated to boiling very cautiously and kept at the boiling point for about five minutes. It was set aside for another five minutes, cooled to room temperature, and shaken with an equal volume of benzene. A bluish-violet to purplish-pink color in the benzene layer was considered a positive test.

6. Isenberg-Buchanan Test

The reagent consists of 25 ml. of concentrated hydrochloric acid in 100 ml. of methanol. In the case of solutions, a volume of reagent equal to the volume of the solution was added and the mixture was allowed to remain at room temperature for 24 hours. For tissue or other solid material, a volume of reagent sufficient to cover the material was added and the reaction was allowed to take place at room temperature for 24 hours. Development of a purple color was considered a positive test.

7. Biuret Test for Proteins

One drop of a 1% solution of cupric sulfate was added to about 0.1 g. of the substance suspended in 5 ml. of 10% sodium hydroxide. Development of a pink to purple color in the solution was considered a positive test.

# APPENDIX III

## ESTIMATION OF LIGNIN CONTENT FROM METHOXYL AND URONIC ANHYDRIDE DETERMINATIONS

Table XXIII on page 115 shows the lignin and methylated uronic anhydride values determined from methoxyl and uronic anhydride figures. As pointed out in the text (page 43), the total uronic anhydride content of a given marc is the sum of the galacturonic and glucuronic anhydrides, each of which has a different methoxyl content. Therefore the values for "uronic anhydride methoxyl" were calculated from the relation,

$$M = 2.2UA/23 + 31U(1 - A)/176,$$

where

M = percent uronic anhydride methoxyl in the tissue being investigated;
U = percent uronic anhydride in the tissue being investigated;
A = fraction of the total uronic anhydride that is galacturonic anhydride (XSc uronic anhydride);
2.2/23 = methoxyl content of the galacturonic anhydride (XSc uronic anhydride); and
31/176 = methoxyl content of the glucuronic anhydride.

The fraction "A" was obtained by reference to Table XV, page 61, in which it can be seen that the galacturonic anhydride (XSc uronic anhydride) accounts for about two-thirds of the total uronic anhydride in the SX tissue and about one-third of the total uronic anhydride in the 54, 53, and whole wood tissues. The ratio "2.2/23" was obtained from Table XXIII, and the ratio "31/176" by assuming that each glucuronic anhydride unit contains one methoxyl group.

TABLE XXIII

LIGNIN CONTENT AND METHYLATED URONIC ANHYDRIDE FROM METHOXYL AND URONIC ANHYDRIDE DETERMINATIONS

| Tissue | Uronic Anhydride, % | Total Methoxyl, % | Uronic Anhydride Methoxyl, % | Lignin Methoxyl, % | Lignin, % | Uronic Anhydride $-CH_2-$, % | Methylated Uronic Anhydride, % |
|---|---|---|---|---|---|---|---|
| XSc | 23.0 | 2.2 | 2.2 | -- | -- | 1.0 | 24.0 |
| SX-1 | 12.5 | 2.6 | 1.5 | 1.1 | 4.5 | 0.7 | 13.2 |
| SX-2 | 11.7 | 2.7 | 1.4 | 1.3 | 5.3 | 0.6 | 12.3 |
| SX-3 | 8.6 | 3.4 | 1.1 | 2.3 | 9.4 | 0.5 | 9.1 |
| SX-4 | 9.6 | 3.2 | 1.2 | 2.0 | 8.2 | 0.6 | 10.2 |
| 54-1 | 6.5 | 4.9 | 1.0 | 3.9 | 15.9 | 0.5 | 7.0 |
| 54-2 | 6.1 | 4.2 | 0.9 | 3.3 | 13.5 | 0.4 | 6.5 |
| 54-3 | 5.5 | 5.2 | 0.8 | 4.4 | 17.9 | 0.4 | 5.9 |
| 54-4 | 5.7 | 5.6 | 0.9 | 4.7 | 19.2 | 0.4 | 6.1 |
| 53-1 | 5.5 | 6.0 | 0.8 | 5.2 | 21.2 | 0.4 | 5.9 |
| 53-2 | 5.1 | 5.6 | 0.8 | 4.8 | 19.6 | 0.4 | 5.5 |
| 53-3 | 5.0 | 5.6 | 0.7 | 4.9 | 20.0 | 0.3 | 5.3 |
| 53-4 | 5.2 | 6.2 | 0.8 | 5.4 | 22.0 | 0.4 | 5.6 |
| Whole wood | 5.6 | 5.9 | 0.8 | 5.1 | 20.8 | 0.4 | 6.0 |

# APPENDIX IV

## ESTIMATION OF METHYLATED URONIC ANHYDRIDE

The values for "uronic anhydride $-CH_2-$" in the preceding table were obtained by multiplying "uronic anhydride methoxyl" by the ratio, 14/31 ($CH_2/OCH_3$). These values were then added to "uronic anhydride" to give the "methylated uronic anhydride" values which appear in Tables XII and XIII.

# APPENDIX V

## COMPARISON OF WHOLE WOOD AND 1953 SAPWOOD

In a recent investigation (29) an aspen (Populus tremuloides) tree was obtained from the same general area as the trees used in this study. An analysis was made of the whole wood, the results of which are presented below together with the values obtained in this investigation for whole wood and 1953 sapwood.

TABLE XXIV

COMPARISON OF TWO WHOLE WOOD ANALYSES WITH 1953 SAPWOOD ANALYSIS[1]

| | Whole Wood This Study | Whole Wood (29) | 1953 Sapwood This Study |
|---|---|---|---|
| Glucan, % | 41.2 | 41.9 | 51.6 |
| Xylan, % | 15.8 | 16.5 | 16.4 |
| Mannan, % | 1.3 | 2.1 | 2.1 |
| Galactan, % | 0.6 | 0.7 | 0.7 |
| Araban, % | 0.3 | 0.6 | 0.5 |
| Uronic anhydride, % | 6.0[2] | 5.0 | 5.6[2] |
| Total carbohydrate, % | 65.2 | 66.8 | 76.9 |
| Lignin, % | 20.8 | 21.2 | 21.3 |
| Protein, % | 0.6 | -- | 0.6 |
| Summation, % | 86.6 | 88.0 | 98.8 |

[1]All calculations were made on the basis of the ovendry, ash-free marcs.

[2]Methylated uronic anhydride.

# APPENDIX VI

## CALCULATION OF GROWTH INCREMENTS

The calculation of growth increments was based on factors derived from the araban figures (see Tables XXVI and XXVII) as follows:

TABLE XXV

FACTORS FOR GROWTH INCREMENT CALCULATIONS

| Growth Interval | Factor |
|---|---|
| XSc--SX | % araban in XSc/% araban in SX = 10.8/2.4 = 4.5 |
| XSc--54 | % araban in XSc/% araban in 54 = 10.8/0.6 = 18.0 |
| XSc--53 | % araban in XSc/% araban in 53 = 10.8/0.5 = 21.6 |

This means that 100 grams of ovendry, ash-, and protein-free XSc tissue will become 450 grams of SX tissue containing carbohydrate and lignin alone.

Similarly, 23.9 grams of glucan at the XSc growth stage (Table XV, page 61) becomes 51.4 x 4.5 = 231 grams at the SX growth stage, 52.4 x 18 = 942 grams at the 54 growth stage, and 52.6 x 21.6 = 1137 grams at the 53 growth stage.

TABLE XXVI

LIGNIN AND CARBOHYDRATE COMPOSITION OF YOUNG ASPEN TISSUES

| Tissue | Lignin, % | Glucan, % | Xylan, % | Mannan, % | Galactan, % | Araban, % | Methylated Uronic Anhydride, % |
|---|---|---|---|---|---|---|---|
| XSc | 0.0 | 15.1 | 6.1 | 0.9 | 10.2 | 6.8 | 24.0 |
| SX | 6.4 | 43.8 | 18.1 | 1.1 | 2.5 | 2.0 | 11.2 |
| 54 | 16.6 | 48.5 | 17.5 | 1.5 | 1.4 | 0.6 | 6.4 |
| 53 | 21.3 | 51.6 | 16.4 | 2.1 | 0.7 | 0.5 | 5.6 |

TABLE XXVII

COMPOSITION OF YOUNG ASPEN TISSUES ON BASIS OF CARBOHYDRATE AND LIGNIN ALONE[1]

| Tissue | Lignin, % | Glucan, % | Xylan, % | Mannan, % | Galactan, % | Araban, % | Methylated Uronic Anhydride, % |
|---|---|---|---|---|---|---|---|
| XSc | 0.0 | 23.9 | 9.7 | 1.4 | 16.2 | 10.8 | 38.0 |
| SX | 7.5 | 51.4 | 21.3 | 1.3 | 2.9 | 2.4 | 13.2 |
| 54 | 18.0 | 52.4 | 18.9 | 1.6 | 1.5 | 0.6 | 6.9 |
| 53 | 21.7 | 52.6 | 16.7 | 2.1 | 0.7 | 0.5 | 5.7 |

[1]All calculations were based on the ovendry, ash-, and protein-free marcs.